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Reactivity of Anti-Group a Type 12 Streptococcal Cell Membrane Monoclonal Antibody to Human Glomerular Basement Membrane

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REACTIVITY OF ANTI-GROUP A TYPE 12 STREPTOCOCCAL
CELL MEMBRANE MONOCLONAL ANTIBODY TO HUMAN
GLOMERULAR BASEMENT MEMBRANE

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
GRADUATE SCHOOL OF LOYOLA UNIVERSITY CHICAGO
IN PARTIAL FULFILLMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

BY

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MAYWOOD, ILLINOIS

MAY, 1991

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CELL MEMBRANE MONOCLONAL ANTIBODY TO HUMAN
GLOMERULAR BASEMENT MEMBRANE

Infection with group A nephritogenic streptococci has long been associated with development of sequelae diseases such as poststreptococcal glomerulonephritis (PSGN). The etiology of PSGN is unknown, but postulated to be mediated by immune complex deposition in the GBM or mediated by antibodies evoked by streptococcal cell membrane (SCM) which react with a shared epitope in the glomerular basement membrane (GBM). A murine mAb was produced against group A type 12 SCM and was hypothesized to react with human renal cortical tissue and isolated GBM. This mAb, IIF4, was examined for reactivity with a panel of 26 human, isolated, enzyme digested GBMs. IIF4 reacted with all digests of GBM digests (trypsin, collagenase or pepsin) equivalently and with all 26 GBMs to a significantly greater extent than an isotype matched mAb did with the GBMs. Two groups of GBMs were indicated when extent of IIF4 reactivity was examined. IIF4 was hypothesized to react with a component of GBM and was established to react with collagen IV, which is a major component of the GBM. IIF4 did not react with fibronectin, laminin or heparan sulfate proteoglycan or collagen I. Attempts were made to identify the location of the epitope on the

collagen IV molecule. Generally, IIF4 reacted with polypeptides of 120 and 100 kDa from collagenase digested GBM or collagen IV and polypeptides of 100 kDa from pepsin digested GBM. These polypeptides were analyzed for amino acid content and each contained a high number of cysteine residues (15-30). The size and cysteine content of these and similarly sized polypeptides from pepsin digested collagen IV was consistent with the IIF4 reactive polypeptide as part of the triple helical domain and the NCl domain and the IIF4 reactive site as a globular portion of a collagen IV polypeptide. The significance of reactivity of an anti-SCM mAb with collagen IV is discussed with regard to molecular mimicry as it pertains to autoimmune disease and development of PSGN.

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LIST OF ABBREVIATIONS

ASPGN	Acute Poststreptococcal Glomerulonephritis
BCA	Bicinchoninic Acid
BSA	bovine serum albumin
ELISA	Enzyme Linked Immunosorbent Assay
GBM	glomerular basement membrane
kDa	kilodalton
ug	microgram
mg	milligram
M	molar
MW	molecular weight
mAb	monoclonal antibody
ng	nanograms
nm	nanometers
O.D.	optical density
PMSF	phenylmethyl sulfonyl fluoride
PBS	phosphate buffered saline
PSGN	poststreptococcal glomerulonephritis
RT	room temperature
SDS	sodium doceyl sulfate
SDS-PAGE	sodium doceyl sulfate polyacrylamide gel electrophoresis
SCM	Group A type 12 streptococcal cell membrane

TEMED N,N,N',N'-Tetramethylethylenediamine
TSB tryptic soy broth
V volts

INTRODUCTION

The clinical association between streptococcal infection and subsequent development of glomerulonephritis has long been established (Futcher, 1940; Rammelkamp, Weaver, and Dingle, 1952; Stollerman, 1969). Streptococci are grouped by surface polysaccharides; in group A, streptococci are serologically typed by surface M protein. Clinically, in the United States, of over 70 M types, ten have been termed nephritogenic (Stollerman, 1969). Infection with one of these types of streptococci can lead to complications of glomerulonephritis. A question that our laboratory would ultimately like to answer is what is the nature of the association between infection with a nephritogenic strain of streptococci and development of glomerulonephritis. Experimental immunochemical evidence, gathered in our laboratory over the last twenty years, has pointed to an antigenic relationship between the cell membrane of group A type 12 streptococci and a component of the glomerular basement membrane (Markowitz and Lange, 1964; Lange, 1980b). In order to examine this question more closely, murine monoclonal antibodies were generated to group A type 12 SCM (Fitzsimons, Weber, and Lange, 1987). Screening and clone selection

was based on the ability of the monoclonal antibody to display reactivity to both pooled glomerular basement membrane antigen as well as streptococcal cell membrane antigen. One of these monoclonal antibodies, IIF4, was chosen for use in this study of the glomerular antigen related to an SCM antigen.

LITERATURE REVIEW

Streptococci are grouped by surface polysaccharide. In beta hemolytic, group A streptococci (the major pathogenic group), streptococci are typed by surface M protein; this work was accomplished by Lancefield (Stollerman, 1975). There are over 70 serologic types of group A streptococci and of these 70, ten have been clinically correlated with nephritis. In the United States, types 1, 2, 4, 12, 18, 25, 49, 55, 57, and 60 are termed nephritogenic (Stollerman, 1969). Type 12 has been most frequently associated with pharyngeal infections leading to glomerulonephritis. This clinical correlation between infection with streptococci and development of glomerulonephritis was first noted by Richard Bright in the early 19th century after a patient developed nephritis following an upper respiratory infection and scarlet fever (Williams, 1987). Subsequently, group A nephritogenic streptococci were implicated in the development of glomerulonephritis, rheumatic fever and skin diseases such as impetigo and scarlet fever following infection of the pharynx or skin (Futcher, 1940; Zabriskie, 1971; Rammelkamp, Weaver, and Dingle, 1952).

Infection with a nephritogenic strain of streptococci and subsequent development of life-threatening nephritis is a rare occurrence in the United States. However, in the developing third

world countries, these sequelae diseases pose serious problems for health care workers and serious health problems for children, especially in tropical climates (Williams, 1987; Rodriguez-Iturbe, 1984; Sorger et al., 1987; Fischetti, 1989).

The M protein classifies the serotype of the streptococci; however, streptococci are defined as nephritogenic if that M type of streptococci is isolated from a patient who developed acute glomerulonephritis (Rodriguez-Iturbe, 1984). It is known that a 10-15% incidence of glomerulonephritis results after nephritogenic streptococcal infection (Anthony et al., 1969; Dillon, 1972) and this attack rate is related to the site of infection. The M protein is also the means by which the streptococci escape the host immune response. M proteins are long fibril proteins projected from the cell wall surface of the streptococci; it is composed of alpha helical coiled coil proteins (Beachey, Seyer, and Kang, 1978; Phillips et al., 1981). The M protein must be opsonized before host phagocytic cells can engulf and destroy the bacteria (Fischetti, 1989; Beachey et al., 1986).

M proteins have been shown to share homology with myosin (Fischetti, 1989; Krisher and Cunningham, 1985; Dale and Beachey, 1985; Jones et al., 1986; Cunningham et al., 1989). M protein was also reported to share an epitope with the intermediary filament vimentin (Kraus et al., 1989) and a 46 kDa component of glomerular tissue (Goroncy-Bermes et al., 1987; Kraus and Beachey, 1988). Clearly, the M protein is a complex protein with several indicated shared epitopes with mammalian tissue.

Although the M protein is responsible for host invasiveness, what defines a streptococci as nephritogenic? This is an unresolved issue (Rodriguez-Iturbe, 1984). Two theories have been generated to explain the role of streptococci in the development of glomerulonephritis. In the first theory, immune complexes between streptococcal components and antibody are formed and then deposited in the GBM (Michael et al., 1966; Friedman et al., 1984). Circulating immune complexes have been isolated from patients with poststreptococcal glomerulonephritis (Van de Rijn et al., 1978; Rodriguez-Iturbe et al., 1980; Jennette et al., 1982). Alternatively, cationic antigens are deposited in the GBM and immune complexes are formed in situ (Vogt et al., 1983; Stinson et al., 1983).

In acute poststreptococcal glomerulonephritis, antibodies are made against streptococci; this is judged clinically by patient serum titers to streptolysin-O, a hemolysin of group A streptococci (Sorger et al., 1983; Williams, 1987). Some investigators have shown the presence of streptococcal antigen in glomeruli from patients with poststreptococcal glomerulonephritis, especially in the acute phase of the disease (Yoshizawa et al., 1973; Anders et al., 1966; Michael et al., 1966; Treser et al., 1970). However, the streptococcal component of the immune complex or the immune complexes in patients with poststreptococcal glomerulonephritis could not always be demonstrated. Furthermore, the nature of the immune complex streptococcal component is controversial (Rodriguez-Iturbe, 1984).

The second theory postulates that a component of nephritogenic streptococci, the cell membrane, and a component of the glomerulus, the basement membrane, share a common antigenic component (Markowitz and Lange, 1964). The theory predicts that antibodies raised to a component of SCM react with GBM. The reaction is antibody mediated and therefore may be classified as potentially autoimmune. This is based on immunochemical evidence which showed that an anti-serum raised to type 12 SCM reacted with extracts of human glomeruli; anti-serum raised to glomerular extract reacted with SCM (Markowitz and Lange, 1964). A chemical relationship between SCM and GBM was indicated by amino acid and carbohydrate analysis of GBM and SCM enzyme digest derived fragments which were found to be antigenically related through the use of anti-SCM and anti-GBM sera (Lange, 1969). These studies indicated that SCM and GBM were related antigenically.

Further evidence of antigenic relatedness was shown with anti-SCM serum reactive with GBM in kidney sections (Markowitz and Lange, 1964; Blue and Lange, 1975). This activity could be absorbed by isolated GBM, or glomeruli, but not with liver or spleen homogenates. Likewise, anti-glomerular serum reacted with GBM in tissue sections and this activity could be absorbed by SCM. These experiments also indicated that the glomerular carbohydrate masked the anti-SCM serum reactive sites on murine GBM, particularly in relatively carbohydrate rich adult tissue as compared to neonatal tissue (Blue and Lange, 1975; Blue and Lange, 1976; Lange, 1980a). In general, young human and murine glomerular tissue was more

reactive with anti-SCM serum in immunofluorescence than adult tissue. Thus, an immunochemical basis was established for the antigenic relatedness of GBM and SCM.

In order to investigate the nature of the antigenic relatedness between GBM and SCM, anti-SCM serum was used as an immunizing agent and a surrogate for streptococcal infections. Guinea pigs injected with anti-SCM serum developed necrotic skin lesions (Lange, 1973). These studies indicated that the anti-SCM serum was responsible for significant skin pathology. Mice injected with anti-SCM serum presented with subepithelial granular deposits on the GBM as judged by electron microscopy and had incorporation of proline into GBM proteins; these proteins were suggested to be a noncollagenous glycoprotein and a collagenous protein from rate of incorporation (Nayyar, Lange, and Borke, 1985). Anti-SCM serum alone was responsible for the skin and renal pathology seen in these two reports.

The pathology seen in human acute poststreptococcal glomerulonephritis is characterized by glomerular infiltration by neutrophils, and the proliferation of mesangial cells. Also, electron dense deposits are located subepithelially or subendothelially on the GBM and the presence of C3 and fibrinogen in an irregular deposition pattern on the GBM is noted (Rodriguez-Iturbe, 1984). Poststreptococcal glomerulonephritis is associated with the expression of HLA-DR1, HLA-B7, and HLA-B44 (Naito, Kohara, and Arakawa, 1987). This may allow classification of poststreptococcal glomerulonephritis with autoimmune diseases such

as ankylosing spondylitis and Reiter's syndrome. Glomerulonephritis can be induced by anti-SCM serum or SCM components. Glomerulonephritis was induced in rhesus monkeys actively by injections of soluble glycoprotein from SCM or passively by injections of antibodies raised against SCM glycoprotein. In both cases, clinical signs of glomerulonephritis were present (Markowitz et al., 1971). Mice injected with anti-SCM serum presented kidney pathology similar to pathology present in kidney biopsies from patients with acute poststreptococcal glomerulonephritis (Nayyar, Lange, and Volini, 1985). Presence of neutrophils, increased cellularity in the mesangium and subepithelial electron dense 'humps' were noted. The evidence indicated a direct action of the anti-SCM serum on the glomerulus.

It can be argued that the use of a serum is not specific enough to detect common antigens in two discrete proteins. Serum contains a mixture of host antibodies directed towards many immunogens. Therefore, a series of murine monoclonal antibodies generated to group A type 12 SCM were produced and mAbs were selected for reactivity with GBM as well as lung basement membrane (Fitzsimons, Weber, and Lange, 1987). Isolated murine or human enzyme digested (trypsin or collagenase) GBM could inhibit binding of several of the mAbs to its autologous antigen, SCM (Fitzsimons, Weber, and Lange, 1987; Zelman and Lange, 1989). These experiments indicated that an antigenic relatedness between GBM and SCM existed based on the definition of an mAb reacting with a unique, specific epitope.

The SCM component containing the epitope shared with GBM has not been identified, but a tryptic digestion fragment of the component was isolated by anti-SCM mAb affinity chromatography. A 13 kDa polypeptide was isolated, but other, 2.3 to 11.2 kDa, polypeptides were noted to bind the anti-SCM mAb affinity column when each was isolated by molecular sieving and then passed over the affinity column. Relative epitope densities were assigned to each polypeptide based on their molecular size and molar ratio of polypeptide bound to mAb. Relative epitope density increased with the size of the polypeptides. This was interpreted to mean that the intact SCM polypeptide consisted of repeating epitopes (Zelman and Lange, 1989).

Evidence based on anti-SCM mAb reactivity with isolated GBM indicated that SCM and GBM shared an epitope. However, the GBM component containing this common epitope was unknown. Basement membranes are complex extracellular matrices composed of several proteins. The functions of basement membranes are compartmentalization of organs, stability of tissues, inhibition of tissue invasion by cells, and filtration of macromolecules. Basement membranes are found in kidney, the blood-brain barrier, placenta, gut, lung, skin, cornea, and blood vessels and can be found encircling muscle, nerve and fat cells (Glanville, 1987). Cells surrounding basement membrane generally produce the membrane and remain in close contact with the membrane; this is thought to be mediated by cellular receptors binding to extracellular matrix ligands (Timpl, 1989).

GBM is a specialized extracellular matrix which serves as a border between the urinary side and the capillary side of the glomerular capillary wall. Capillary endothelial side cells are a continuous sheet of cells punctuated by fenestrae. Urinary epithelial cells have characteristic podocyte morphology and the foot processes are separated by filtration slits. Mesangial cells face the remaining parts of the membrane. Ultrastructurally, the GBM is composed of three layers; the lamina rara externa (urinary side), the lamina densa (slightly more electron dense than the other layers), and the lamina rara interna (capillary side) (Wieslander, 1983; Timpl, 1989). Both the endothelial and epithelial cells are known to produce extracellular matrix which fuses to form the basement membrane (Timpl and Aumailley, 1989).

The specialized function of the GBM is to ultrafilter the blood plasma to begin the process of urine formation. The GBM has an overall negative charge and allows proteins of less than 70 kDa to filter into the urine (Vogt et al., 1983; Timpl, 1989). The electrostatic charge of the plasma proteins is important. Proteins of greater than 70 kDa with a pI of greater than 8.5 can accumulate in the GBM and form in situ immune complexes with circulating antibody (Vogt et al., 1982). The integrity of the GBM is a crucial matter; immune complexes or antibody deposited in the GBM can lead to an inflammatory response of complement activation, neutrophil chemotaxis and lysosomal enzyme release and damage to the GBM leading to proteinuria and renal failure in some cases (Rodriguez-

Iturbe, 1984). Thus, the integrity of the GBM is an important matter.

The components of the GBM are collagen IV (which is a three chain coil) composed of alpha 1(IV) and alpha 2(IV) chains, laminin which is composed of an A, B1 and B2 chains, nidogen or entactin, heparan sulfate proteoglycan, BM-40 and possibly other components. (Timpl, 1989; Timpl and Aumailley, 1989).

Collagen IV is the major component of the GBM comprising between 30% and 80% of its total mass. Collagen IV derived from human placental membrane and bovine capsular membrane is well characterized. Collagen IV is believed to provide the structural network for the GBM (Kuhn et al., 1981; Timpl, 1989). Collagen IV is a distinct collagen type found only in basement membranes and is distinctly different from classical fibril-type interstitial collagens I, II, and III (Kefalides, 1973). Collagen IV is the only type of collagen (eleven types have been identified) that forms non-fibrillar networks (Kuhn, 1987). All collagens contain three alpha chains which are composed of repeating triplet of glycine-X-Y which account for the helical structure of the molecule. Hydroxylated proline and lysine are found interspersed in the X-Y positions and are only found in collagen molecules (Kuhn, 1987).

Collagen IV differs from classical collagens in two major characteristics. First, collagens are synthesized as a procollagen precursor with globular domains at the amino and carboxyl termini of the alpha chain. Three alpha chains wound in a triple helix form procollagen. In classical collagens, the globular domains are

cleaved by extracellular enzymes and are not incorporated into the collagen molecule or fibril. However, in types IV and VI collagen, these globular domains are retained. Second, collagen IV has 21 small, globular interruptions in the three alpha chains; these are believed to allow for flexibility of the collagen molecule in basement membrane assembly (Kuhn, 1987; Glanville, 1987).

Mature collagen IV is composed of three alpha chains (each with amino and carboxyl globular domains) wound into a superhelix. There are four forms of the type IV alpha chain: alpha 1(IV), alpha 2(IV), alpha 3(IV) and alpha 4(IV). Classically, collagen IV molecules were thought to be composed of two alpha 1(IV) and one alpha 2(IV) chain although it is not known if all collagen IV molecules in a basement membrane will be composed similarly. Collagen IV molecules have been identified with one alpha 1(IV) chain and two alpha 2(IV) chains (Timpl, 1989; Brazel *et al.*, 1988). In addition to this heterogeneity, alpha 3(IV) chains have been identified in GBM with alpha 1(IV) and alpha 2(IV) chains. The occurrence of alpha 3(IV) chains is now thought to be wide spread (Timpl, 1989; Hudson *et al.*, 1989). This greatly increases the potential heterogeneity of the collagen IV molecule in a GBM.

The human alpha 1(IV) chain, which is 1669 amino acid residues in length, is composed of a signal peptide (amino acids 1-27), the amino terminal overlap and crosslink 7S globular domain (28-172), the central alpha helical region (173-1440) and the noncollagenous NC1 domain (1441-1669) (Brazel *et al.*, 1988). Alpha 1(IV) chains from mouse and *Drosophila* have also been amino acid

sequenced as well as alpha 2(IV) in human and mouse. All alpha chains have some homology with each other (Timpl, 1989). The 7S domain of each alpha chain has a high percentage of hydrophobic amino acids as well as 4 cysteine residues and two lysine residues; these are responsible for inter- and intramolecular crosslinks with other alpha chains and other collagen IV molecules. Another cysteine residue (position 98) is exclusively involved in intermolecular crosslinks. The number and position of cysteine residues are conserved in human alpha 1(IV) and alpha 2(IV) chains (Glanville, 1987). The NC1 domain of an alpha chain has a high percentage of hydrophobic amino acids and a total of 12 cysteine residues per domain. Two homologous subregions (35% homology with an additional 21% conservative substitutions) each contain 6 cysteine residues (Glanville, 1987, Timpl, 1989).

The function of the two major globular domains of collagen IV in basement membrane formation are aggregation (7S domain) and dimer formation (NC1 domain). Two models for the assembly of the collagen IV backbone in basement membranes have been proposed; the network and the hexagonal model (Yurchenco, 1990; Timpl, 1989). These two theories are based on rotary shadowed electron microscopy of isolated collagen IV molecules. In the network assembly model, the intact 7S domain consists of the aggregation and overlap of 7S domains of 4 collagen IV molecules (12 alpha chains total) and the dimer formation of NC1 domains from two collagen IV molecules. This assembly model leads to a chicken wire-like network of assembled collagen IV molecules. In the hexagonal model, self assembly is

initiated by NCl dimer formation, followed by association of 12 collagen molecules (36 alpha chains) (Yurchenco, 1990; Glanville, 1987). Regardless of the correctness of either model, the end result is a highly disulfide-stabilized, cross-linked association of 7S to 7S domains and NCl to NCl domains. This forms the backbone of the basement membrane.

Another major GBM component is laminin. Murine laminin is best characterized. Laminin (800 kDa) is composed of three disulfide-linked polypeptides chains: B1 and B2 (200 kDa each) and A (400 kDa with isoforms). These chains are folded into a cross-like structure with 3 short arms and one long arm. Each arm has globular and rod-like domains with marked alpha helical domains at the carboxyl terminus of each arm. Each of the 4 arms are homologous in 1200 amino acids at their amino termini. The short arms are cysteine rich and have similarity to epidermal growth factor. In addition, the long arm has a unique globular domain at its carboxyl terminus distal to the junctional region of the cross (Timpl and Aumailley, 1989; Ekblom et al., 1990; Abrahamson et al., 1989). Laminin has several binding and biologic properties. Nidogen, a 150 kDa single polypeptide chain with a dumbbell shape, is stably, noncovalently bound to laminin in an equimolar ratio with the short arm of laminin (Timpl, 1989; Mann et al., 1989). Nidogen may be identical to entactin, a sulfated 15 kDa protein found in cell cultures (Mann et al., 1989). It has been suggested that nidogen/entactin mediates laminin binding to collagen IV (Timpl, 1989). On the opposite short arm from nidogen, is a cell binding

site; another cell binding site is located on the globular end of the long arm. Although this has not been proven for laminin, the cell binding sequence, arginine-glycine-asparagine (RGD) has been demonstrated in collagen IV. The RGD sequence mediates cell binding in fibronectin, vitronectin, von Willebrand factor, osteopontin, integrins, nidogen and collagen I (Timpl and Aumailley, 1989; Pierschbacher and Ruoslahti, 1984; Mann et al., 1989). The globular domain of the long arm has also been implicated in heparin binding (Timpl, 1989; Martin and Timpl, 1987). Like collagen IV, laminin molecules can polymerize which may contribute to the assembly of the basement membrane (Yurchenco and Furthmayr, 1984).

The major proteoglycan in GBM is heparan sulfate proteoglycan; murine and human heparan sulfate proteoglycan have been characterized. There are two forms of heparan sulfate proteoglycan: low and high buoyant density. The protein core for the low density form (30% by weight carbohydrate) is 480 kDa with three heparan sulfate chains attached to it. The heparan sulfate chains (in human) are each 18 kDa and are anionic (Van Den Heuvel et al., 1989; Timpl, 1989; Schleicher et al., 1989). Polyanionic heparan sulfate proteoglycan is believed to give the basement membrane the majority of its negative charge. Small heparan sulfate proteoglycans (high density) have also been identified in the GBM; these star-shaped molecules (130 kDa) have a protein core with four heparan sulfate chains attached (Edge and Spiro, 1987; Timpl, 1989). Chondroitin sulfate proteoglycan and dermatin sulfate proteoglycan have been identified in small amounts in the GBM by chemical

characterization and immunochemical localization (Paulsson et al., 1985).

Formation of the basement membrane is dependent on divalent cations, especially calcium. Laminin polymerization is calcium dependent (Yurchenco and Furthmayr, 1984) and the association of laminin and nidogen is calcium dependent (Paulsson et al., 1987). Another calcium dependent basement membrane protein is BM-40 (also called osteonectin or SPARC) which is a single chain 35 kDa protein. BM-40 binds calcium and is ubiquitous in basement membranes (Timpl and Aumailley, 1989).

In total, the GBM is a complex structure of heterogeneous collagen IV, laminin, nidogen/entactin, heparan sulfate proteoglycan with small amounts of other proteoglycans, BM-40 and possibly other unidentified components. The exact percentages of each component in a basement membrane are unknown and, in the GBM, may vary with the age of the individual. Also, the exact mechanism of GBM assembly is unknown and therefore the ultrastructural localization of each component is not fully understood (Yurchenco, 1990; Yurchenco et al., 1986). The importance of the integrity and maintenance of function of the GBM is, however, indisputably critical.

Important characteristics of the GBM are its antigenicity and immunogenicity. In experimental nephritis, a proliferative glomerulonephritis can be induced by injection of heterologous GBM into sheep (Stebly, 1962). This evidence along with many other reports (Unanue and Dixon, 1965; Dixon, Feldman, and Vasquez, 1961; Hammer and Dixon, 1963; Hoedemaeker et al., 1972), established the

antigenicity and immunogenicity of GBM and its ability to initiate nephrotoxic disease. This experimental system attempts to parallel the conditions in some human glomerulonephritis. Patients with glomerulonephritis possess antibodies reactive with GBM (Wieslander, Bygren and Heinegard, 1983; Wilson and Dixon, 1974; Lerner, Glasscock, and Dixon, 1967) although the exact target of these antibodies is not determined (Wieslander, Bygren and Heinegard, 1983). In Goodpasture syndrome, which has glomerular and pulmonary hemorrhagic involvement, autoantibodies are directed to a specific component of collagen IV (Wieslander et al., 1984; Kefalides et al., 1986; Pusey et al., 1987). Serum from a Goodpasture patient reacts specifically with the NC1 portion of the alpha 3 (IV) chain (Hudson et al., 1989). The specific portion of the NC1 domain reactive with Goodpasture sera is a 26 kDa monomer (designated M2*) which is a noncollagenous polypeptide believed to be sequestered within the hexameric structure of the NC1 domain. There is also reactivity to an apparently nonreduced 52 kDa dimer (Wieslander, Kataja, and Hudson, 1987; Pusey et al., 1987; Weber, Meyer zum Buschenfelde, and Kohler, 1988). Reactivity of the Goodpasture serum to collagenase digested collagen IV or basement membrane from bovine lens capsule, lung, or placenta or human placenta, lung, or glomeruli indicated that the Goodpasture antigen is widespread (Weber et al., 1987; Fish et al., 1984; Weber, Meyer zum Buschenfelde, and Kohler, 1988; Wieslander, Kataja, and Hudson, 1987). It has been speculated that the Goodpasture antigen is sequestered, masked or otherwise privileged and only revealed after dissociation of the NC1 hexamer

by toxic, infective or other traumatic episode (Weber, Meyer zum Buschenfelde, and Kohler, 1988). The Goodpasture autoantigen is the most studied of the defined glomerular antigens.

Another GBM antigen involved in an autoimmune disease, IgA nephropathy, has been suggested to be part of the alpha helical portion of collagen IV. Patient IgA antibodies react with the triple helical portions of the alpha chains (reactivity was destroyed by collagenase digestion) of collagen IV, collagen I and collagen II after pepsin digestion (Cederholm et al., 1986). The autoantigen in familial Alport syndrome involves collagen IV. It has been found that the basement membrane in Alport patients do not have a 'normal' 28 kDa monomer integrated in the NC1 domain. Evidence points to a different NC1 monomer in the alpha 3(IV) chain that is the autoantigen (Kleppel et al., 1989). This autoantigen is closely related to the Goodpasture antigen (Savage et al., 1986). Thus, collagen IV has been identified as the repository of several autoantigens highly associated with specific autoimmune diseases.

The purpose of this thesis is to determine the reactivity of an anti-SCM mAb with human GBM. The association of nephritogenic streptococcal infection and development of glomerulonephritis has been established along with the autoantigenic qualities of several GBM antigens. This thesis seeks to investigate the immunochemical link between SCM and GBM by examining the reactivity of anti-SCM mAb with a panel of human GBMs and to identify the anti-SCM mAb reactive component in GBM.

METHODS AND MATERIALS

Production of Ascites From Mice

Ascites containing monoclonal antibody were produced by standard methods (Johnstone and Thorpe, 1982). Pristane (2,6,10,14 tetramethylpentadecane, Aldrich Chemicals, Milwaukee, WI) primed Balb/c mice (Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with 1×10^6 to 1×10^7 cloned hybridoma cells. Hybridoma cells were produced by the fusion of spleen cells from Balb/c mice immunized with group A type 12 streptococcal cell membrane and P3-X63-Ag8 nonsecreting plasmacytoma cells (Fitzsimons, Weber and Lange, 1987). Twelve to fourteen days post injection, mice were sacrificed by ether inhalation, jugular vein severing and exsanguination. Blood, ascites and hybridoma cells were recovered and heart, kidney and lung tissues collected for examination.

Isotyping of Ascites and Sera

Isotyping of ascites and sera was performed via ELISA according to manufacturer's instructions (Isotyping kit, HyClone, Logan, UT). Briefly, 96 well plates (Becton Dickinson, Oxnard, CA) were sensitized with goat anti-mouse immunoglobulins and then blocked with 1% BSA in PBS. Ascites or sera (0.1 ug/ml protein of

each) were added to wells; a 1:500 dilution of normal mouse serum and normal rabbit serum were used as positive and negative controls, respectively. After washing, goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM monoclonal (as supplied by HyClone) antibodies were added to the plate. Bound antibodies were detected with alkaline phosphatase labeled goat anti-mouse immunoglobulins and a paranitrophenyl phosphate based substrate (both were supplied by HyClone). Optical density of individual wells was read at 405 nm with a microplate reader (MR-600, Dynatech, Alexandria, VA). Reactions were considered positive when above 0.600 optical density.

Euglobulin Precipitation of Ascites

Euglobulin fractionation of ascites is a quick and gentle process to isolate IgM from sera or ascites (Garcia-Gonzales, et al., 1988). Ascites was placed in dialysis tubing, 10,000 molecular weight cutoff (Union Carbide, Chicago, IL), and dialyzed versus distilled water for 18 hours at 4°C. Recovered material was then centrifuged (Eppendorf, Brinkmann) for 3 minutes. The recovered precipitate was solubilized in borate buffered saline, pH 8.5 (0.1 M boric acid, 25 mM sodium tetraborate, 75 mM NaCl) at 4°C.

Quantitation of Murine IgM

For ELISA, 96 well plates were sensitized with goat anti-mouse IgM (0.1 ug/ml) (HyClone) in 0.05 M carbonate buffer, pH 9.6 for 18 hours at room temperature (RT) and then blocked with 1% bovine serum albumin in phosphate buffered saline (PBS), pH 7.4 for

3 hours. After washing, dilutions of the sera, ascites or euglobulin fractions were applied to the plate. TEPC 183 (murine myeloma, IgM, Sigma, St. Louis, MO) was applied to the plate in dilutions of 20 ug/ml to 0.016 ug/ml. After incubation and washing, goat anti-mouse IgM conjugated to peroxidase was applied to the plate at 0.1 ug/ml. After incubation and washing, substrate [equal parts of 0.02% 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid, Sigma) in 0.1 M citrate buffer, pH 4.1 and 0.01% H₂O₂ in PBS] was applied to the plate and the optical density read at 405 nm in the microplate reader at 30 minutes. A standard curve of TEPC 183 concentration versus optical density was generated and the IgM concentration of the sera, ascites or euglobulin fractions was read from this curve.

Quantitation of Murine IgG1

Quantitation of anti-collagen type IV monoclonal antibody (mAb) was accomplished using MOPC 21 (murine myeloma, IgG1, Sigma) and the same methodology as previously described for murine IgM except that peroxidase labeled goat anti-mouse IgG was used as the detecting antibody and the 96 well plate was sensitized with 0.1 ug/ml goat anti-mouse immunoglobulins in 0.05 M carbonate buffer, pH 9.6.

Protein Determination

The bicinchoninic acid (BCA) protein determination assay is based on the interaction of protein and copper ion (Cu⁺²) in alkaline solution to produce cuprous ion (Cu⁺¹); this ion interacts

with BCA to form a colored reaction product. Bovine serum albumin (BSA) (1000 ug/ml to 15.7 ug/ml) are applied to a 96 well plate along with dilutions of the unknown. The BCA reagents are applied to the plate according to the manufacturer (Pierce Chemicals, Rockford, IL) and incubated at 37°C for 30 minutes. Absorbance was read at 560 nm in the microplate reader. A standard curve of BSA concentration was produced and the concentrations of the unknowns were read from the curve.

Examination of Tissue for Antibody Binding

Human kidneys (whole or sections) were obtained from National Diabetes Research Interchange, Philadelphia, PA. All tissues were negative for HIV and Hepatitis B virus. Tissues were collected upon autopsy and no tissue was used in this study from any individual with overt kidney disease. Age at time of death was 56 ± 19 years; this study used the tissue from 16 males and 11 females. Cortical sections were embedded in O.C.T. compound, quick frozen, and held at -70°C until use.

Cryostat Sectioning of Frozen Tissue

Tissue sections embedded in O.C.T. compound were mounted on brass buttons and warmed to -30°C in the cryostat (International Equipment Co., Needham, MA). Sections of 3 microns were cut and dried onto methanol cleaned and Histostik (Accurate Chemical, Westbury, NY) coated glass slides. Tissue sections were fixed onto the slides with acetone for 5 minutes and then washed three times in

PBS. Tissue sections were kept in a moist chamber for immunofluorescence processing.

Immunofluorescence Staining of Tissues

Tissues were examined for localization of antibody binding by indirect immunofluorescence (Johnson, Holbrow, and Dorling, 1978). Tissue sections were treated by direct application of ascites, sera, euglobulin fraction or isolated monoclonal antibody (IIF4, NMS, TEPC 183 [IgM]; anti collagen IV mAb, MOPC 21 [IgG1]) at 10 ug, 5 ug, and 2.5 ug per section in 1% BSA for one hour at RT. After three 5 minute PBS washings, the appropriate conjugate antibody [fluorescein conjugated goat anti-mouse IgM, fluorescein-conjugated goat anti-mouse immunoglobulins; both at 10 ug Ig/ml (HyClone, Logan, UT)] in 0.1% BSA was applied to the sections for one hour at RT. After washing, cover slips were placed on the slides with buffered glycine containing ortho-phenylenediamine as a fluorescence quencher (Johnson and de Nogueira Araujo, 1981). Sections were viewed with a fluorescent microscope (Leitz, Midland, Ontario, Canada).

Photography of Immunofluorescently Stained Tissue

Photographs were taken of immunofluorescently stained tissue sections with the camera attachment on the Leitz microscope. Black and white film (Tri-X Pan, Kodak, Rochester, NY) and color slide film (Ektachrome, Kodak) (each at ASA 400) were exposed for 30 seconds. Black and white negatives were developed in Microdol (Kodak) diluted 1:2 for 10 minutes at 20°C, washed, fixed (Kodak fixer) for 15

minutes at 20°C, washed and air-dried. Prints were made on F4 paper (Kodak), developed in Dektol (Kodak) at 1:2 at 20°C for 1 minute, washed, fixed (Kodak fixer) for 10 minutes, washed and air dried.

Growth of GS-5 Streptococcus mutans

S. mutans (GS-5) (provided by Dr. Brian Shearer, Department of Microbiology, LUMC) 50 ml feeder cultures were grown in sterilized tryptic soy broth (TSB, BBL, Beckton-Dickinson, Cockseyville, MD) with 2.5 mg/ml yeast extract (BBL) at 37°C with shaking overnight (Stinson et al., 1983). Cultures were shown to contain one bacteria type by aliquots streaked on blood agar plates (Baxter Scientific Products, McGraw Park, IL) and incubation at 37°C for two days. Feeder cultures were added to TSB (2 L) and incubated at 37°C with shaking for 6 hours. At this time, 50 ml of filter sterilized 5% glucose (Sigma) and 50 ml of sterilized 5% bicarbonate (Mallinckrodt, Inc., St. Louis, MO) were added to the cultures to boost growth. After 24 hours of incubation, bacteria were collected by centrifugation at 4424 x g (J2-21 Centrifuge, Beckman, Oxnard, CA) for 10 minutes and washed with PBS-0.05% NaN₃ (Sigma) until the supernatants read <0.2 O.D. at 278 nm. Bacteria were heat killed at a density of 10% in PBS-azide at 80°C for 15 minutes with shaking. Total culture killing was checked by aliquots streaked on blood agar plates incubated at 37°C for two days.

Preparation of Group A Type 12 Streptococci

Whole cells of heat killed group A type 12 streptococci (previously prepared in this laboratory) were washed repeatedly in saline at 17699 x g (Beckman) until the supernatants read <0.2 O.D. at 278 nm (Model 139, UV-Vis Spectrophotometer, Hitachi Perkin-Elmer, Danbury, CT).

Preparation of Bacterial Cell Membrane

Bacterial cells were disrupted with the BeadBeater cell disrupter (Biospec Products, Bartlesville, O.K.) using 0.1 mm diameter glass beads previously washed in 50% sulfuric acid and several volumes of distilled water. The BeadBeater chamber was one-third filled with glass beads, bacteria, distilled water and surrounded with ice. Two volumes of glass beads to one volume of bacteria was used for maximum bacterial breakage. The cells were disrupted by a five minute on, five minute off cycle repeated five times (Bleiweis, Karakowa and Krause, 1964). Examinations of Gram stains and wet mounts under phase contrast confirmed presence disrupted cells. Differential centrifugation isolated bacterial cell membrane. Centrifugation at 4424 x g for 10 minutes pelleted whole cells and some cell wall; centrifugation at 6370 x g for 10 minutes pelleted cell wall and centrifugation at 34957 x g for 10 minutes pelleted bacterial membranes. Membrane preparations were dialyzed (3000 molecular weight cutoff, Sephrapor, Spectrum Medical Industries, Inc., Los Angeles, CA) against distilled water and lyophilized (Freezemobile 12, The Virtis Co., Gardiner, NY).

The isolation of group A type 12 streptococcal cell membrane was carried out by Mark Zelman in this laboratory; his preparations are used throughout this study.

Rhamnose Determination of Cell Membrane

The procedure of Dische and Shettles (1946) was used to determine rhamnose (methyl pentose) level in the membrane preparations. A level of <0.1% is acceptable for purified group A type 12 streptococcal cell membrane (Markowitz and Lange, 1964). 4.5 ml chilled 87.5% sulfuric acid was added to ice cold solutions of methyl pentose (Mann Research Laboratories, New York, NY) (2.75, 5.50, 8.25, 10.0 ug/ml) in 1 ml saturated benzoic acid with constant shaking in an ice bath. After standing 10 minutes at RT, the tubes were placed in boiling water for 3 minutes. After cooling to RT, 0.1 ml of 3% cysteine hydrochloride monohydrate was added to each tube. After two hours at RT, the absorption was read at 396 nm and 430 nm. A blank containing 1 ml saturated benzoic acid, 4.5 ml 87.5% sulfuric acid and 0.1 ml cysteine hydrochloride monohydrate was read. The difference in the absorptions (396 nm - 430 nm) gave the rhamnose concentration (ug/ml). The principle of this assay is based on the absence of methyl pentose absorbance at 430 nm. Hexoses, pentoses, glucuronic and galacturonic acids have symmetrical absorption curves such that the absorbance at 430 nm and 396 nm is the same. Subtraction of the absorbance at 430 nm from the absorbance at 396 nm corrects for all the absorbance except for that due to methyl pentose. The membrane and cell wall samples were

first digested in 87.5% sulfuric acid by boiling for 3 minutes. The percent rhamnose was determined by examining the standard curve and assigning a concentration to the unknown.

$$\% \text{ rhamnose} = \frac{\text{ug rhamnose in sample}}{\text{ug membrane in assay}} \times 100$$

ug membrane in assay.

Cell membrane prepared from group A type 12 streptococcal cell membrane was judged to be free of cell wall contaminants by determining the rhamnose concentration of the preparation. The trypsin digested SCM was found to have less than 0.08% rhamnose; this is considered free of cell wall contaminants (Markowitz and Lange, 1964; Lange, 1980a).

Cell membrane was isolated from S. mutans. The yield of S. mutans cell membrane was 0.135 grams. A 9.5 gram wet weight is equivalent to 2-4 grams dry weight of bacteria. Bacterial membrane is approximately 10% of the cell. The minimal expected weight of isolated membrane is 0.2 grams; the yield of S. mutans cell membrane is approximately 67.5%.

Trypsin Digestion of Bacterial Cell Membrane

Lyophilized SCM or S. mutans cellular membrane was weighed and 1 mg trypsin (Sigma) in PBS, pH 6.0, was added per 100 mg membrane preparation (Markowitz and Lange, 1964; Lange, 1969). This preparation was rotated at 37°C for 18 hours, centrifuged at 483 x g for 10 minutes and the supernatant was dialyzed against distilled water overnight at 4°C with a membrane cutoff of 3000 molecular weight (Sephraphor). The supernatant was concentrated to a small vol-

ume (Speed-vac, Savant Instruments, Inc., Hickville, NY). The concentrated, digested membrane was stored at -20°C until use.

Isolation of Human Glomeruli

Glomeruli were isolated as previously described (Greenspon and Krakauer, 1950; Markowitz and Lange, 1964; Blue and Lange, 1975). Human kidneys were weighed and cortical tissue was separated from medullary tissue with scissors. Cortices were then forced through a 64 μ mesh screen into a watch glass on ice adding cold PBS with 30 μM tannic acid (Aldrich Chemicals, Milwaukee, WI) and the nonspecific protease inhibitor, PMSF (phenylmethylsulfonyl fluoride; 1 nM final concentration, Sigma), as needed to facilitate the procedure. Cortical material was collected from the watch glass and allowed to settle in cold PBS with tannic acid and PMSF and the supernatant was aspirated. This was repeated six times yielding a relatively pure preparation of whole glomeruli free of other cells and debris; this was checked by phase contrast microscopy. Glomerular basement membrane was prepared from isolated glomeruli (Carlson et al., 1978). The glomeruli were then osmotically shocked with distilled water with 0.05% NaN_3 and PMSF overnight at RT with gentle shaking to free the glomerular basement membrane (GBM). The preparation was centrifuged for 10 minutes at 483 x g and the supernatant was aspirated. 3% Triton-X 100 was added and the tubes were rotated for 2-4 hours. The preparation was centrifuged as above and the pellet treated with 2000 units of DNase in 1 M NaCl and gently shaken and incubated for 1-2 hours at 37°C . The prepara-

tion was centrifuged and the pellet treated with 4% deoxycholate for 2 hours at RT. After centrifugation, the pelleted material was washed 5 times in azide-water. The recovered sediment was judged by microscopic review to be >95% purified glomerular basement membrane and was stored at -20°C until digestion. This procedure yields a preparation that is enriched for glomerular basement membrane.

Enzyme Digestion of Human GBM or Collagen

Enzymatic digestions of membrane preparations were essentially as previously described (Markowitz and Lange, 1964; Lange, 1969). Some modifications are as follows.

Glomerular basement membrane (GBM) sediment, collagen type I (Sigma) or collagen type IV (Sigma) was weighed and 1 mg bacterial collagenase (Sigma) in PBS, pH 7.4, with 0.01 mM calcium lactate and 1 mM PMSF was added per 100 mg of GBM sediment or collagen. Preparations were rotated slowly for 18 hours at 37°C and then centrifuged at 483 x g for 30 minutes. Supernatants were stored at -20°C until use.

Glomerular basement membrane, collagen I, or collagen IV was weighed and 1 mg of trypsin (Sigma) in PBS, pH 6.0, was added per 100 mg sediment or protein. Preparation were rotated for 18 hours at 37°C and then centrifuged at 483 x g for 10 minutes. Supernatants were stored at -20°C until use.

GBM, collagen I, or collagen IV were pepsin digested (Qian and Glanville, 1984; Glanville et al., 1985). GBM sediment (GBM 18509 N, 18274 N) or protein was digested by adding 0.02 milligrams

of pepsin (Nutritional Biochemicals Corporation, Cleveland, OH) per milligram of material to be digested in 0.5 M formic acid (pH 2.0) at 4°C with mixing for 16 hours. After neutralization with 10 N NaOH, the mixture was centrifuged and stored at -20°C until use.

Amino Acid Analysis

Protein, isolated polypeptide or whole collagenase or trypsin digested glomerular basement membrane was digested to single amino acids with constant boiling 5.7 N HCl in sealed N₂ flushed, evacuated tubes for 22 hours. Preparations were dried a desiccator containing NaOH pellets which absorb HCl from the sample. Dried preparations were dissolved in sample buffer and analyzed (Beckman 6300 Amino Acid Analyzer) using an ion exchange column and lithium buffers and a program that compares amino acid composition of the preparation to a standard separation of amino acids. Amino acid composition was converted to percent amino acid per 100 amino acid and total weight (microgram) of preparation using a spreadsheet (Lotus 1-2-3, Cambridge, MA).

Enzyme Linked Immunosorbant Assay (ELISA)

Proteins, polypeptides, or tissue isolates were tested for reactivity to anti-SCM mAb by ELISA (Voller, Bidwell and Bartlett, 1980). Plates (96 wells) were sensitized with 5 ug/ml of the material to be assayed in 0.05 M carbonate buffer, pH 9.6, overnight at RT. Wells were blocked with 1% BSA in PBS for two hours at RT. Dilutions of a known antibody concentration of mAb, serum or ascites

were applied to the plates for one to two hours at RT. For IgM primary antibody, dilutions of 20 ug/ml to 0.078 ug/ml (100 ul/well) were applied to the plates in triplicate. For IgG1 primary antibody, dilutions of 45 ug/ml to 0.01 ug/ml were applied to the plates. After extensive washing with PBS-0.05% Tween 20, enzyme conjugated detector antibody was added to the wells (100 ul/well) for one hour at RT. A peroxidase-labeled goat anti-mouse IgM antibody (HyClone, Logan, UT) and a peroxidase labeled goat anti-mouse IgG antibody (HyClone) were used at 0.1 ug/ml in 1% BSA-PBS for IgM and IgG1 primary antibodies, respectively. After extensive washing, substrate was applied to the wells (150 ul/well) and allowed to develop for 30 minutes and then read with a microplate reader at 405 nm. For peroxidase conjugated detector antibody, equal parts of 0.02% 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) (Sigma) in 0.1 M citrate buffer, pH 4.1 and 0.01% H₂O₂ in PBS were used as substrate. Absorption was read at 405 nm at 30 minutes. Mean optical density and standard deviation were calculated for each reading and plotted versus ug/ml of antibody.

Competitive Inhibition Studies of IIF4 Reactivity

A. Streptococcal cell membranes as competitors

Dilutions of trypsin digested SCM or trypsin digested S. mutans cell membrane (final concentration: 40 ug/ml to 0.1 ug/ml) were added with IIF4 (final concentration: 2.5 ug/ml) in PBS and incubated at 37°C for two hours. After centrifugation, 100 ul/well was applied to a trypsin digested SCM (0.5 ug/ml) sensitized and BSA

blocked 96 well plate. After a one hour incubation and extensive washing with PBS-0.05% Tween 20, plates were processed as described in ELISA and tested for unbound antibody on plates sensitized with trypsin digested SCM or pepsin digested collagen IV. These assays were performed with at least three replicates and each experiment was performed at least three times.

B. Fibronectin, heparan sulfate proteoglycan, laminin, and bovine serum albumin as competitors

Laminin, and heparan sulfate proteoglycan are major components of GBM along with collagen IV. Native fibronectin (human serum; Boehringer Mannheim, Indianapolis, IN), native laminin (derived from Englebreth-Holm-Swarm Mouse sarcoma; Sigma, St. Louis, MO.), heparan sulfate proteoglycan (derived from bovine kidney; Sigma) and bovine serum albumin (Sigma) were diluted in PBS (final concentrations, 1.0 to 0.00001 ug/ml) were incubated with IIF4 (final concentrations, 2.5 ug/ml) at 37°C for two hours and then processed as above.

C. Enzyme digests of collagen I, collagen IV, and GBM as competitors

This assay was used to determine IIF4 reactivity with the major constituent of GBM, collagen IV, and as a comparison, collagen I and with GBM. Dilutions of collagenase digests of GBM and human placenta collagen IV (Sigma) and pepsin digests of human placenta collagens I and IV (final concentration, 10 to 0.0001 ug/ml) were

incubated with IIF4 (final concentration, 2.5 ug/ml) at 37°C for two hours and then processed as described above.

Competitive Inhibition of anti-collagen IV mAb reactivity

As a comparison for competitive inhibition of IIF4 reactivity, the anti-collagen IV mAb reactivity was competitively inhibited by pepsin digested human placenta collagens I and IV, fibronectin, and trypsin digested SCM. Dilutions of SCM, pepsin digested collagens I and IV and fibronectin (final concentration, 10 to 0.00001 ug/ml) were incubated with anti-collagen IV mAb (final concentration, 0.5 ug/ml) for two hours at 37°C and then processed as described in ELISA except that the antibody-inhibitor mixture was applied to 96 well plates sensitized with trypsin digested GBM at 0.5 ug/well and peroxidase labeled goat anti-mouse IgG at 0.1 ug/ml was used as the secondary antibody.

SDS-PAGE of Proteins

Bacterial membrane preparations, glomerular basement membrane preparations, isolated proteins, or immunoprecipitated complexes were separated on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) (Lammeli, 1970). A 10% or 15% gel (10% or 15% acrylamide, 1% N-N methylene bis-acrylamide crosslinker, Kodak) was polymerized using ammonium persulfate (1.7 mM final concentration) and TEMED (0.52 mM final concentration) (Sigma). Stacking gels of 5% were used. Proteins to be applied to gels were denatured by incubating at 90°C for 5 minutes in SDS-sample buffer (0.06 M Tris, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3%

SDS, pH 6.8). Proteins of known molecular weight were used as standards (Bio-Rad, Richmond, CA.): phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Proteins were electrophoresed through the stacking gel at 70 V and through the separating gel at 175 V. The runs were terminated when the bromophenol blue dye (1% aqueous) included in the standard molecular weight protein mixture reached the bottom of the gel.

Staining of and Duplicating Protein Gels

Gels were stained with Coomassie Brilliant Blue R-250 or were silver stained (described below). Gels were stained in 10% Coomassie Brilliant Blue R-250 (Sigma) in 50% trichloroacetic acid (Sigma) for 1-2 hours with agitation. Gels were destained in 7.5% acetic acid and 5% methanol for several hours. Coomassie stained gels were duplicated by positive photographic images with EDP Electrophoresis Duplicating Paper (Kodak). Under a 1A red safelight filter, EDP paper was placed emulsion side up on a flat surface. An amber filter (supplied by the manufacturer) was placed between the EDP paper and the gel to be duplicated. A 15-watt incandescent light source was placed 36 inches over the EDP paper/filter/gel assembly and the paper was exposed for 15-20 seconds. EDP paper was developed in Dektol at 1:1 for 2 minutes at RT, washed and then fixed (Kodak fixer) for 2 minutes at RT, washed for 2 minutes and then air dried.

Elution of Protein from SDS-PAGE

Protein preparations containing higher molecular weight proteins (100-140 kDa) of interest were separated on 6.5% SDS-PAGE, stained with Coomassie Brilliant Blue and destained. Stained bands of interest were cut from the gel and minced into small (1 mm³) pieces. Gel pieces were washed in distilled water and then equilibrated in 0.05 M NH₄CO₃, 0.1% SDS for 2 hours at RT. After equilibration, gel pieces were placed on the cathode side of a lucite chamber; chamber halves were separated by a dialysis membrane (3000 MW cutoff, Sephrapor). The lucite chamber was cooled at all times in an ice bath to prevent overheating of the gel pieces. After 50 milliamperes were applied for approximately two hours, supernatant was recovered from both halves of the chamber, dialyzed against 20% methanol overnight at 4°C, dialyzed against distilled water for 2 hours at 4°C, and lyophilized. Protein in eluted material was quantitated and then subjected to amino acid analysis. Recovery of protein from SDS-PAGE, elution and dialysis was poor, in the range of 15-20%. This was determined by comparing the density of the polypeptide band to the total density of the total protein in the lane.

Immunoprecipitation

In order to specifically detect proteins or polypeptides reactive with anti-SCM mAb, immunoprecipitation was done (Chen et al., 1986; Weir, 1979). Immunoprecipitation of proteins reactive with anti-SCM mAb was carried out using goat anti-mouse IgM coupled

to agarose beads (Sigma). The beads will bind a maximum of 0.4 mg IgM per ml of bead. The agarose beads were washed extensively with PBS. GBM, SCM, or other protein aliquots were preincubated by treatment with pre-washed beads for one hour with mixing at 37°C. The SCM, GBM or other protein aliquots were recovered by centrifugation. To couple mAb to the beads, either anti-SCM mAb, IIF4, TEPC 183, or IgM from the euglobulin fraction of normal mouse serum was reacted with the pre-washed beads at 0.5 mg/ml for 18 hours at 4°C with gentle mixing. The beads are then washed four times in 4 volumes of cold PBS to remove unbound mAb. Protein to be precipitated (SCM, GBM or other protein) was added to the bead/mAb complex at 1 mg/ml (100 ug total) and incubated at 4°C for 18 hours with gentle mixing. After three washings in four volumes of PBS, precipitated material was eluted from the beads with 0.1 acetic acid, 0.15 M NaCl, pH 2.4 for 3-5 minutes with gentle mixing. Eluted material was recovered by centrifugation, neutralized with 10 N NaOH, dialyzed against distilled water for 15 minutes and concentrated to a small volume (approximately 30 ul). Eluted material was then denatured in SDS sample buffer and applied to 10% or 15% SDS-PAGE. After electrophoresis, gels were silver stained.

Preclearing of Immunoprecipitable Material

In order to demonstrate specificity of the immunoprecipitation reaction, a preclearing step was added to the above protocol. Protein that was preincubated with the agarose beads was subjected to immunoprecipitation as described above with

IIF4 or TEPC 183 conjugated beads. The supernatant of the immunoprecipitation reaction was reacted again with fresh IIF4- or TEPC 183-conjugated beads. Material was eluted and processed as described in the immunoprecipitation section.

silver Staining of SDS-PAGE

Gels were silver stained in order to detect small amounts of protein (Gooderham, 1984). After electrophoresis, gels were pre-fixed in 50% methanol overnight. Gels were fixed in 10% glutaraldehyde for 30 minutes with agitation. Gels were washed free of excess glutaraldehyde with four distilled water washes of 30 minutes each. Gels were treated with ammoniacal silver. 8.0 ml of 0.073 mM AgNO_3 was added to a solution of 93% 0.1 N NaOH and 7% NH_4OH (45 ml total). This was diluted to 200 ml (total) and added to a gel for 20 minutes with rocking, washed twice with distilled water and then developed (0.1 grams of citric acid, 0.1 ml of 37% formaldehyde in 200 ml distilled water) for 20 minutes. Gels were held in 50% methanol until photographed.

RESULTS

Anti-SCM mAb is IgM Isotype

After production of murine monoclonal antibodies to group A type 12 SCM (Fitzsimons, Weber and Lange, 1987), one of the mAbs was isotyped. As positive controls, a 1:500 dilution of normal mouse serum, 0.5 ug/ml each of murine myeloma TEPC 183 mAb (IgM), murine myeloma MOPC 21 mAb (IgG1), and anti-collagen IV mAb (IgG1) were used. The isotypes of TEPC 183, MOPC 12 and anti-collagen IV were previously known from the supplier and were used as monoclonal controls for the isotyping system. As demonstrated in Table 1, all mAbs with previously known isotypes reacted as expected; normal mouse serum contains all isotypes and all isotypes were detected. As a negative control, a 1:500 dilution of normal rabbit serum was used; since this is a murine isotyping system, no murine isotypes were detected in normal rabbit serum. The anti-SCM mAb, IIF4, was isotyped as IgM.

Reactivity of IIF4 with SCM and S. mutans Cell Membrane

This experiment was performed to demonstrate the reactivity of anti-SCM mAb, IIF4, with its autologous antigen, group A type 12

TABLE 1.

Isotype of Murine Monoclonal Antibodies

<u>Monoclonal</u>	<u>Isotype</u>
Anti-collagen IV	IgG1
MOPC 21	IgG1
TEPC 183	IgM
IIF4	IgM
<u>Control sera</u>	
normal mouse serum	+ all isotypes
normal rabbit serum	- all isotypes

All monoclonal antibodies were tested at 0.5 ug/ml; sera were tested at a dilution of 1:500.

Euglobulin fraction of ascites IIF4 was tested.

A positive isotype was an optical density > 0.600 at 405 nm. Isotypes of anti-collagen IV mAb, TEPC 183, and MOPC 21 were previously known and used as controls.

SCM and compare this reactivity to S. mutans cell membrane. In an ELISA, 0.5 ug of either trypsin digested SCM or S. mutans cell membrane were reacted with equal amounts of IgM in IIF4, TEPC 183, or normal mouse serum (5 ug/ml to 0.313 ug/ml). In Figure 1, IIF4 reacted with SCM and not another streptococcal cell membrane, S. mutans membrane (at 5.0 and 2.5 ug/ml IIF4, $p < 0.001$). TEPC 183 and IgM from normal mouse serum did not react with SCM (at 5.0 and 2.5 ug/ml IgM, $p < 0.001$). Reactions of IIF4 and TEPC 183 with the cell membrane from S. mutans were not statistically different (at 5.0 and 2.5 ug/ml IgM, $p > 0.05$). This result indicates that anti-group A type 12 SCM mAb reacted with its autologous antigen, group A type 12 SCM. S. mutans cell membrane will be used as a negative control for SCM.

Competitive Inhibition of IIF4 Reactivity with SCM and S. mutans Cell Membrane as Inhibitors

IIF4 reactivity, as presented in Figure 1, with its autologous antigen, SCM and not with another streptococcal cell membrane from S. mutans was confirmed by competitive inhibition.

Trypsin digested SCM, at 5000 ng, inhibited IIF4 reactivity to a maximum of 50%. Trypsin digested cell membrane from S. mutans did not inhibit IIF4 reactivity to any extent distinguishable from background (Figure 2). This result confirmed and demonstrated that IIF4 reacted with its autologous antigen, SCM, and not another streptococcal cell membrane from S. mutans.

Figure 1. Reactivity of IIF4 with Trypsin Digested Group A Type 12 SCM and Trypsin Digested Cell Membrane from S. mutans

IIF4, TEPC 183 and IgM from NMS (serial dilutions of 5.0 to 0.313 ug/ml IgM) were applied to 96 well plates sensitized with 5.0 ug/ml of either trypsin digested SCM or trypsin digested S. mutans cell membrane. After application of peroxidase labeled goat anti-mouse IgM at 0.1 ug/ml and peroxidase based substrate, color development was recorded at 405 nm at 30 minutes.

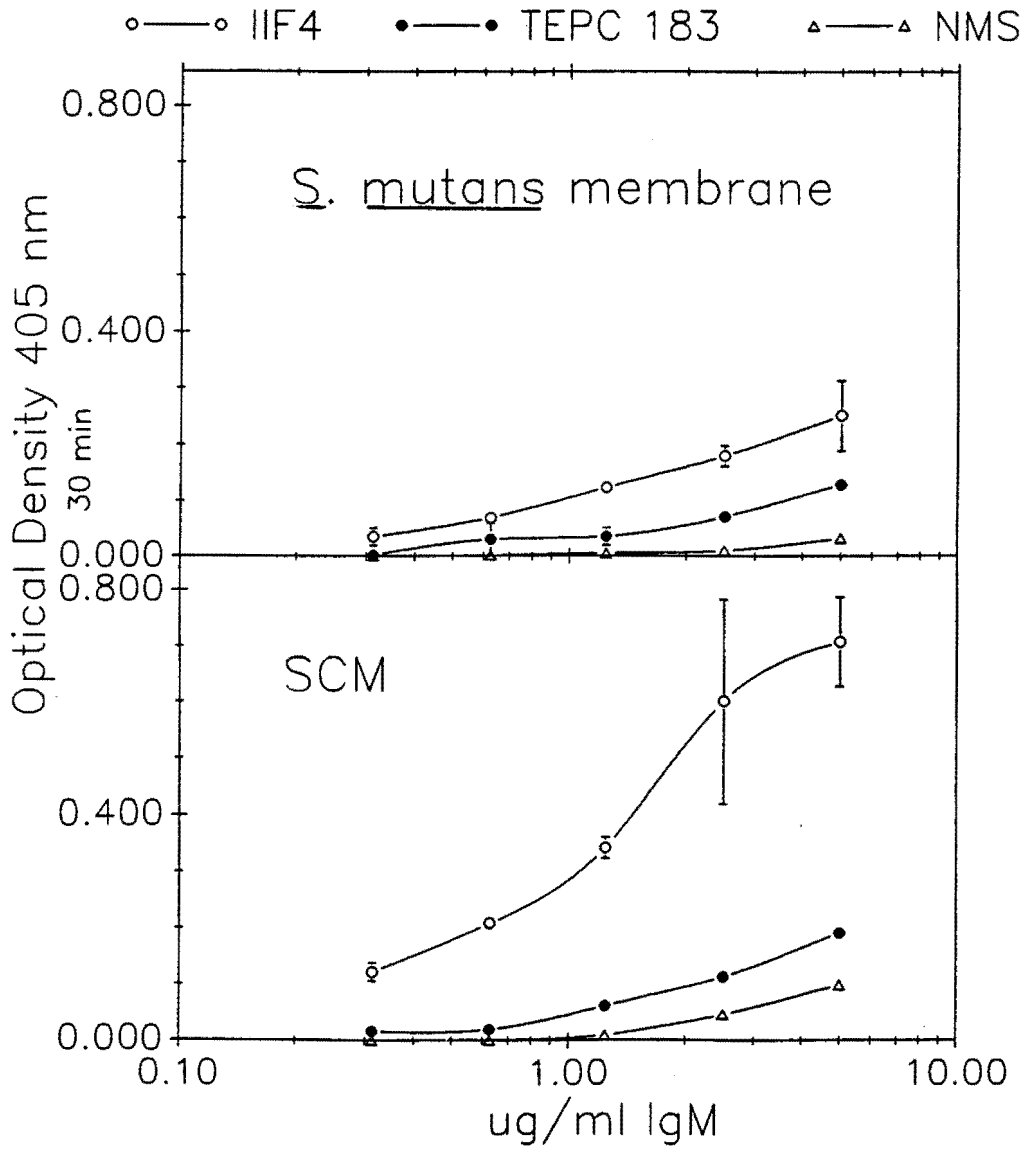
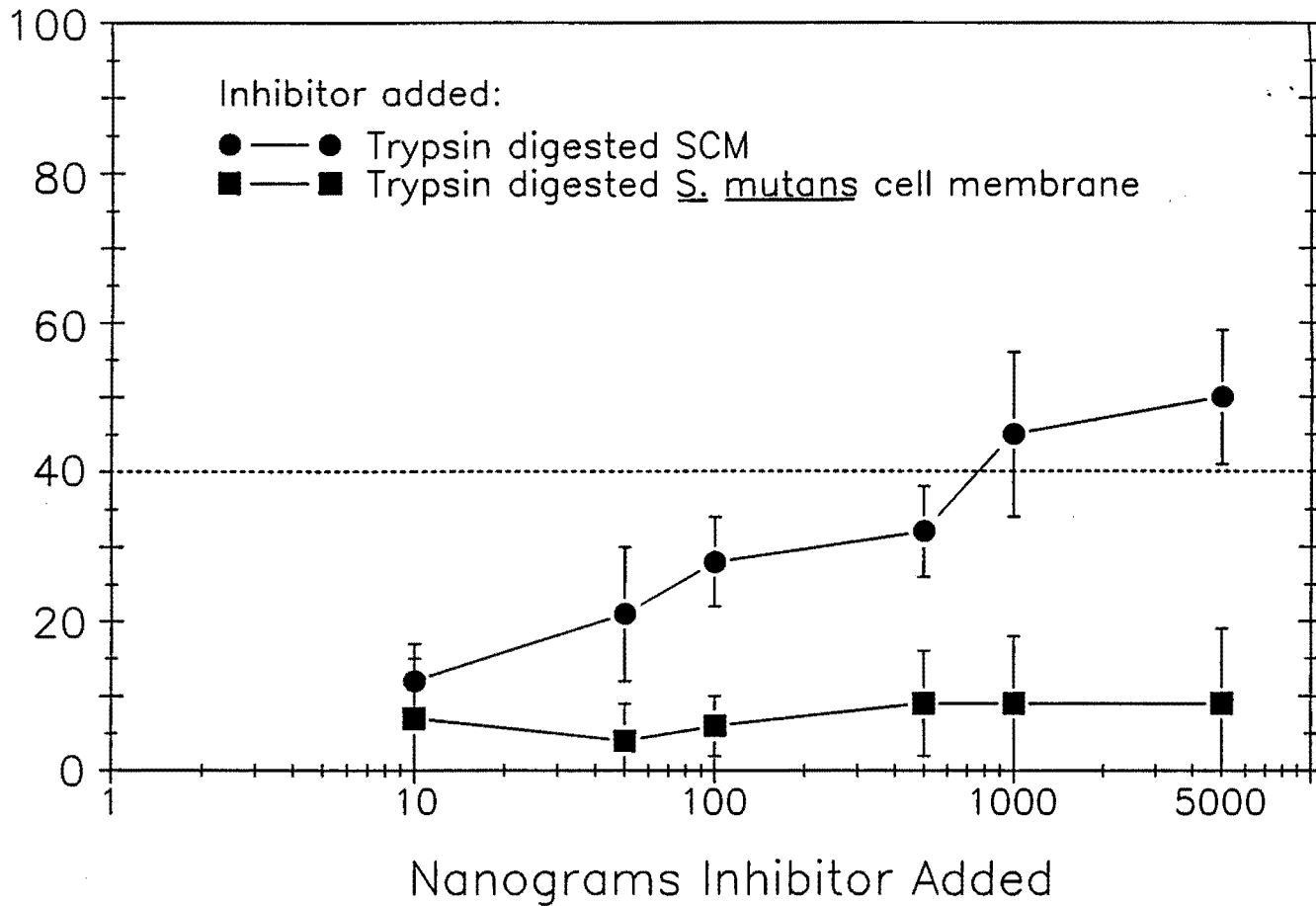


Figure 2. Competitive Inhibition of IIF4 Reactivity with Bacterial Cell Membranes

IIF4 reactivity with trypsin digested SCM (5.0 ug/ml bound to a 96 well plate) was competitively inhibited with trypsin digested SCM, but not trypsin digested S. mutans cell membrane. IIF4 (2.5 ug/ml) was reacted with dilutions of SCM or S. mutans cell membrane for two hours at 37°C. Aliquots (100 ul/well) were then applied to trypsin digested SCM plates. After processing as described in METHODS, color development was recorded at 405 nm at 30 minutes. Each assay was performed a minimum of three times with a minimum of three replicates for each point and the average and standard deviation of IIF4 reactivity is presented.

Percent Competitive Inhibition
of IIF4 Reactivity
(at 2.5 ug/ml)



Anti-SCM mAb Binding is Localized to GBM

IIF4 was hypothesized to recognize an epitope in human GBM. Cortical tissue from human kidneys (18274 N, 19442 N, 19226 N, 19763 N) was assayed for binding of anti-SCM mAbs. Cortical tissue treated with TEPC 183 showed no binding of antibody on indirect immunofluorescence. However, equal amounts of anti-SCM mAb IIF4 showed immunofluorescence of GBM in the cortical tissue preparations examined (Figure 3). A granular pattern of immunofluorescence was consistently seen in all cortical tissue examined. Nuclei of mesangial cells were autofluorescent (yellow-orange in color). Some immunofluorescent staining was also seen of tubular basement membrane, mesangial matrix, and vessel walls.

Amino Acid Analysis of 25 GBMs

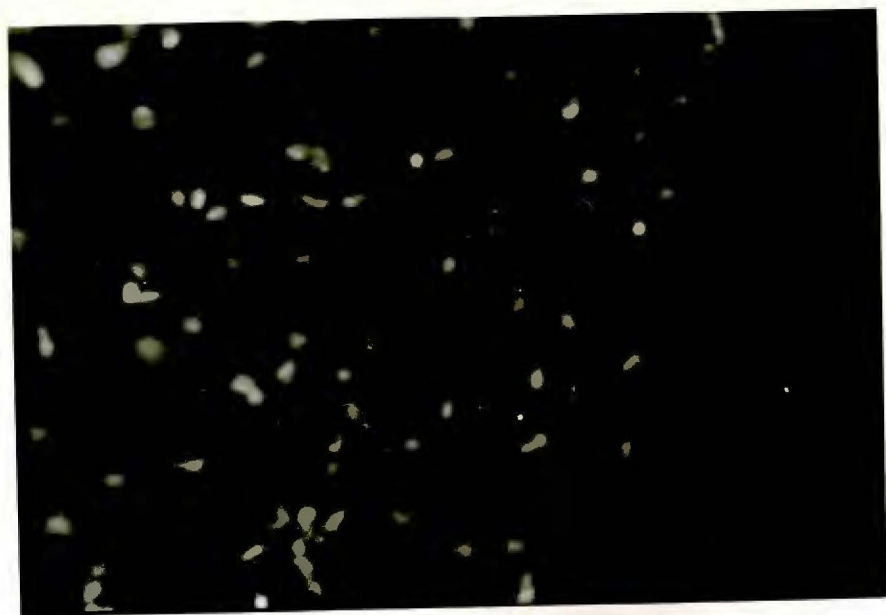
Twenty five human GBMs were analyzed for amino acid composition. In Table 2, the compiled percentage of each amino acid in the GBMs is given. The presence of hydroxyproline and hydroxylysine in the 25 GBMs established these GBMs as containing collagen; only collagen contains these amino acids.

Reactivity of Anti-SCM mAb with Isolated Human Glomerular Basement Membrane (GBM)

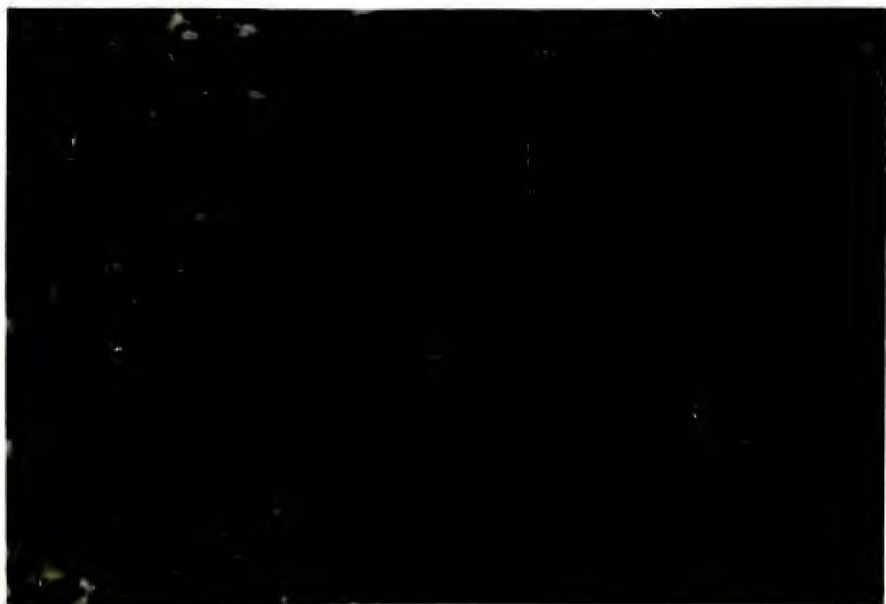
IIF4 was hypothesized to react with isolated, human GBM. To test this, GBMs were isolated as described from 26 human kidneys. 5 ug of isolated, solubilized GBMs were individually coated

Figure 3. Indirect Immunofluorescence of Human Renal Cortical Tissue with IIF4 and TEPC 183

Cryostat sectioned human renal cortical tissue was treated with IIF4 or TEPC 183 (10 ug of IgM in 1% BSA) for one hour at RT. After washing, tissue sections were incubated with fluorescein-conjugated goat anti-mouse IgM for one hour at RT, washed and viewed on a fluorescent microscope. Panel A is IIF4 treated cortical tissue; IIF4 bound to GBM in a granular pattern, mesangial matrix, and tubular basement membrane. TEPC 183 (Panel B) did not bind to the cortical tissue. Mesangial nuclei were auto-fluorescent.



B



A

TABLE 2.

Amino Acid Composition of
Human Glomerular Basement Membrane
(percent amino acid)

Amino Acid	Percent Amino Acid (mean +/- SD)
Cysteic acid	0.98 +/- 0.99
Aspartic acid	8.86 +/- 0.48
<u>Hydroxyproline</u>	<u>3.62 +/- 1.32</u>
Methionine sulfoxide	0.57 +/- 0.31
Threonine	4.53 +/- 0.58
Serine	4.32 +/- 0.52
Glutamic acid	12.73 +/- 0.84
Proline	6.85 +/- 0.61
<u>Glycine</u>	<u>13.57 +/- 2.50</u>
Alanine	4.70 +/- 0.41
Hexosamines	0.16 +/- 0.15
Valine	4.66 +/- 0.76
Cysteine	0.45 +/- 0.37
Methionine	0.84 +/- 0.41
Isoleucine	4.04 +/- 0.42
Leucine	7.85 +/- 0.85
Tyrosine	3.27 +/- 0.46
Phenylalanine	4.25 +/- 0.19
<u>Hydroxylysine</u>	<u>2.43 +/- 0.72</u>
Lysine	4.47 +/- 0.60
Histidine	2.20 +/- 0.26
Arginine	4.76 +/- 0.85

Twenty five human, isolated GBMs were analyzed for amino acid content. Each GBM was analyzed twice. The mean is the mean of the means for the 25 samples for each amino acid.

SD-- standard deviation.

on 96 well plates and reacted with IIF4 and TEPC 183. In Figure 4, the individual results of 26 GBMs reactivity with IIF4 and TEPC 183 at 5.0 ug/ml IgM are compiled and shown. As represented in this paired data, all GBMs reacted with to a greater extent with IIF4 than TEPC 183 ($p < 0.01$, at minimum significant difference). The GBM with the least difference in reactivity between the levels of IIF4 and TEPC 183 reactivity displayed significantly greater reactivity with IIF4 (at 5.0 and 2.5 ug/ml IgM, $p < 0.02$, in both cases). At 5.0 ug/ml IgM, the mean of the means of the GBMs for IIF4 reactivity was significantly greater than the mean of the means for TEPC 183 (at $p < 0.001$). These data indicated that all of the GBMs reacted preferentially with IIF4 as compared to TEPC 183. These data further indicated that IIF4 reacted with isolated human GBM and reacted with all GBMs tested.

IIF4 Reactivity with Trypsin or Collagenase Digested GBMs

Since IIF4 was shown to react with isolated GBMs, reactions of IIF4 with trypsin digested GBMs and collagenase digested GBMs were examined. IIF4 reacted to an equal extent with trypsin digested GBMs and collagenase digested GBMs (Figure 5). In this paired data, the mean of the means at 5.0 ug/ml IIF4 indicated no difference in IIF4 reactivity to the two types of enzyme digested GBMs ($p > 0.2$ for trypsin versus collagenase digested GBMs). TEPC 183 reactivity with trypsin digested GBMs was less than with collagenase digested GBMs; reactions of TEPC 183 with collagenase digested GBMs

Figure 4. IIF4 Reactivity with 26 Human GBMs

Isolated, trypsin digested or collagenase digested human GBMs were reacted with IIF4 and TEPC 183 in ELISA. Plates were sensitized with 5.0 ug/ml of the GBM and reacted with IIF4 or TEPC 183 (10 to 0.05 ug/ml IgM) for one hour at RT. After washing, plates were processed as described in METHODS and MATERIALS. Each GBM was assayed in triplicate at least three times. Reactivity of the GBMs at 5.0 ug/ml of IIF4 and TEPC 183 is shown as paired data.

26 GBMs (either collagenase or trypsin digested)
tested at 5.0 $\mu\text{g}/\text{ml}$

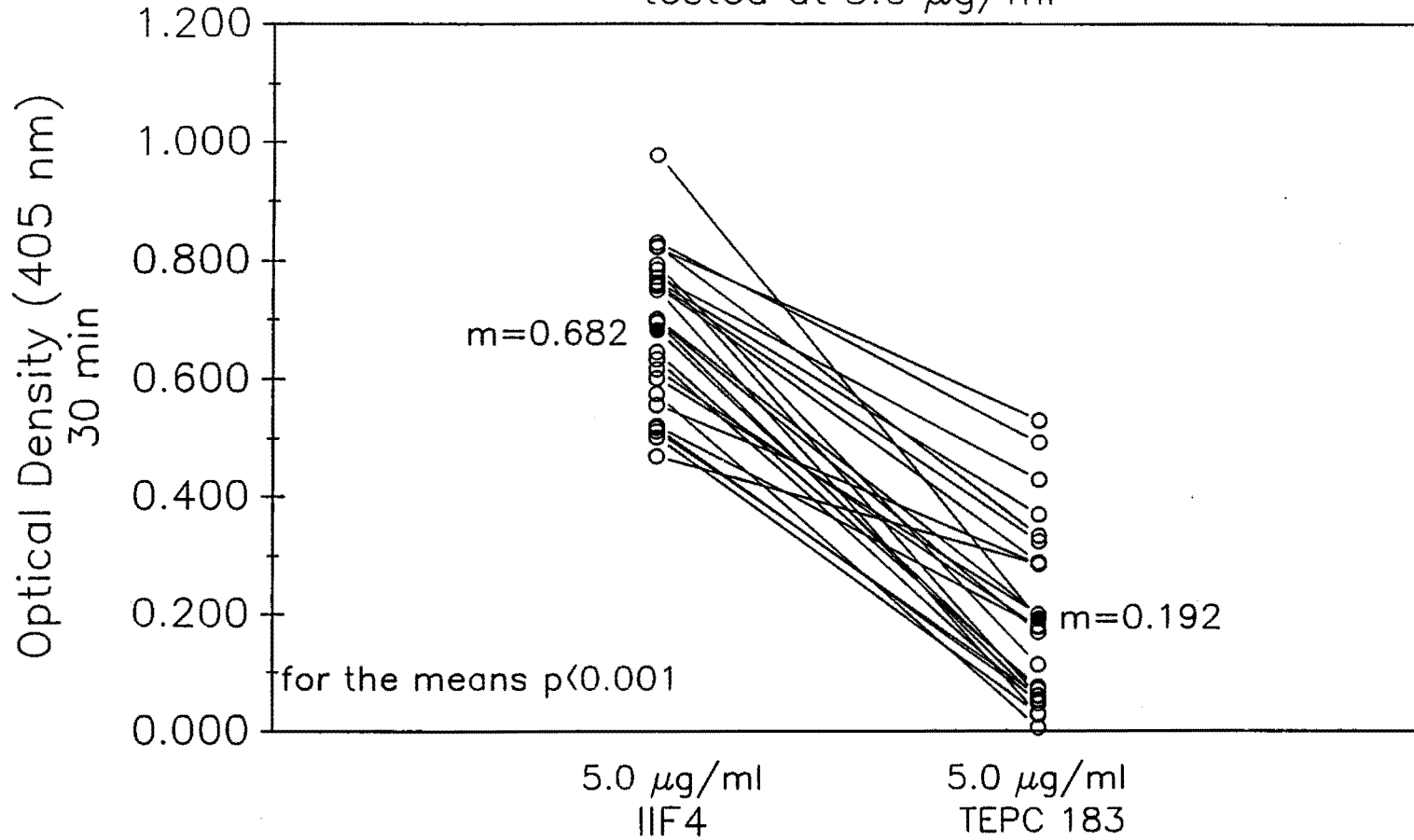


Figure 5. IIF4 does not React Differently to Trypsin and Collagenase
Digested GBMs

IIF4 reactivity of trypsin versus collagenase digests of GBM was examined at 5.0 ug/ml of IIF4 and TEPC 183. Reactions of TEPC 183 with trypsin digested and with collagenase digested GBMs are also shown.

GBMs tested at 5.0 $\mu\text{g/ml}$

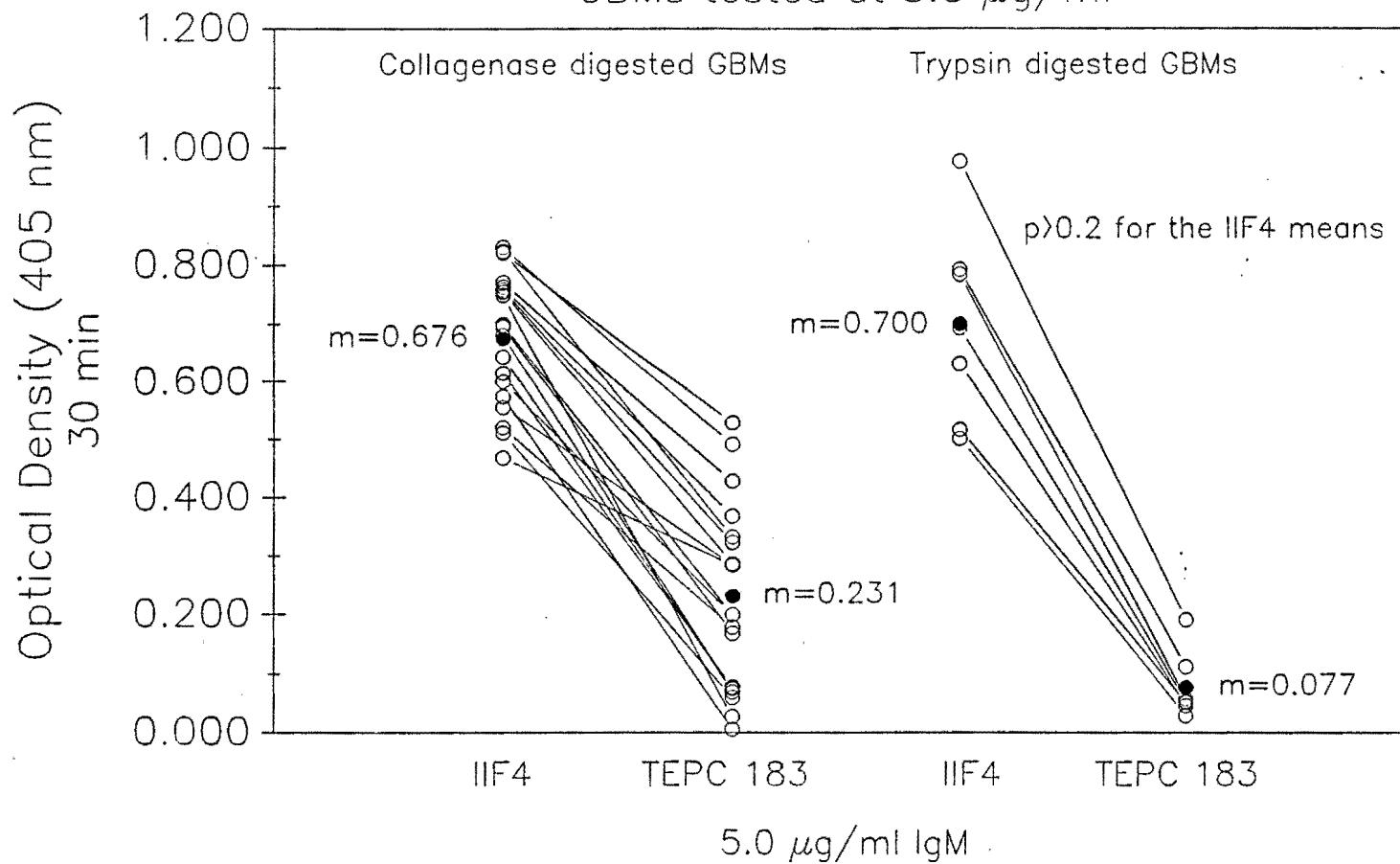
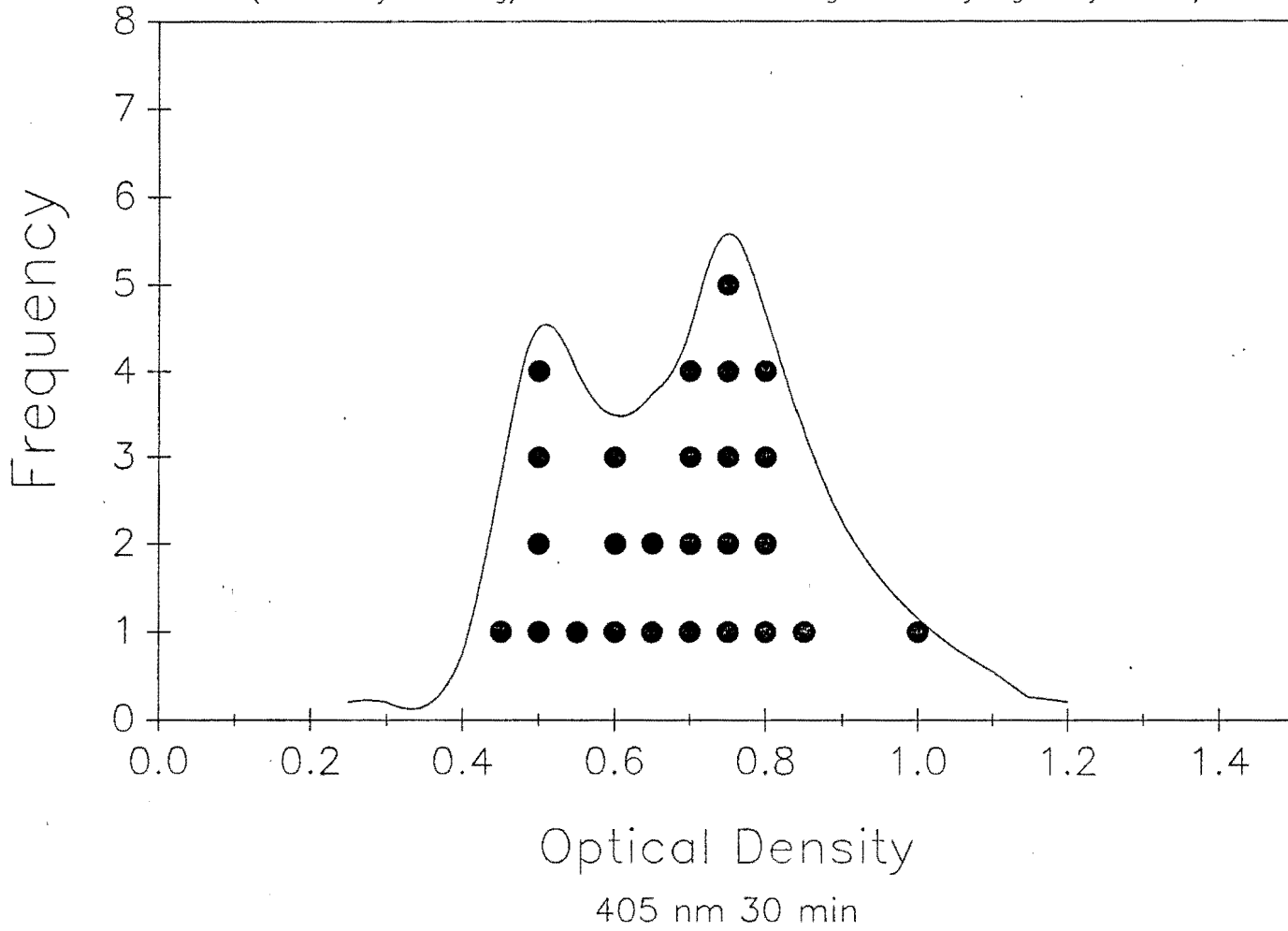


Figure 6. Bimodal Distribution of IIF4 Reactivity with 26 GBMs

IIF4 reactivities at 5.0 ug/ml with the GBMs were placed in optical density groups of 0.05 and plotted for frequency of occurrence without regard to standard error. There are two groups of GBM with respect to extent of IIF4 reactivity when reactivity is examined in this manner.

(reactivity of 5 ug/ml of IIF4 with 0.5 ug of GBM judged by ELISA)



were greater than with trypsin digested GBMs, but reactivity of TEPC 183 with collagenase digested GBMs is significantly less than IIF4 reactivity with collagenase digested GBMs (at 5.0 ug/ml IgM, $p < 0.05$). However, the range of TEPC 183 reactivity with trypsin digested GBMs was encompassed by the range of TEPC 183 reactivity with collagenase digested GBMs. These results indicate that TEPC 183 reactivity in this system was variable and was considered nonspecific. These results demonstrated that IIF4 reacted equally well with trypsin and collagenase digested GBMs; this strongly suggested that the IIF4 epitope exists in both types of enzyme digested GBM preparations.

Bimodal Distribution of IIF4 Reactivity with 26 Human GBMs

Values for IIF4 reactivity at 5.0 ug/ml IgM with 26 human trypsin or collagenase digested GBMs were placed in 0.05 O.D. groups and plotted for frequency occurrence without regard to standard error of the reactivity of each GBM with IIF4. As shown in Figure 6, two groups of GBMs were evident. These data suggested that two groups of GBM, one with higher IIF4 reactivity and one with lower IIF4 reactivity, existed in the population of 26 GBMs examined.

Comparison of IIF4 Reactivity among Bacterial Cell Membranes, Isolated Proteins Comprising GBM, and GBM

Anti-SCM mAb, IIF4, was hypothesized to recognize an epitope common to SCM and GBM. It is useful to compare IIF4 reactivity with SCM, proteins comprising the GBM and the GBM itself so that one may

begin to understand how IIF4 reacts with preparations thought to contain the common epitope. Figure 7 compares IIF4 reactivity among these protein preparations.

Figure 7, panel A represents data in which trypsin digested SCM reacted to a significantly greater extent than trypsin digested S. mutans cell membrane (at 5.0 and 2.5 ug/ml IgM, $p < 0.001$) (Figure 1). In Panel B, IIF4 and TEPC 183 reactivity among collagenase, trypsin, or pepsin digests of GBMs is represented. In this sample of IIF4 reactivity with enzyme digested GBMs, all GBM preparations reacted with IIF4 to a similar extent; these results are examined in more depth in Figure 8. All enzyme digested GBMs reacted with IIF4 to a significantly greater extent than with TEPC 183 (for each preparation, at 5.0 and 2.5 ug/ml IgM, $p < 0.01$, at minimum significant difference).

Panel C represents IIF4 reactivity with collagenase digested collagen I and collagenase digested collagen IV, and native laminin. IIF4 did not react with laminin to any extent significantly different from TEPC 183 reactivity with laminin (at all antibody concentrations, $p > 0.2$). However, collagenase digested collagen I and collagenase digested collagen IV did react with IIF4 to a significantly greater extent than with TEPC 183 (for both preparations, at all antibody concentrations, $p < 0.001$).

Panel D represents IIF4 reactivity with pepsin digested collagen I or collagen IV, trypsin digested collagen I and native fibronectin. IIF4 did not react with fibronectin to any extent greater than with TEPC 183 (at 5.0 and 2.5 ug/ml IgM, $p > 0.05$). IIF4

did react with pepsin digested collagen I and pepsin digested collagen IV to a significantly greater extent than the digests did with TEPC 183 (at 5.0 and 2.5 ug/ml IgM, $p < 0.01$, at minimum significant difference). Trypsin digested collagen I also reacted with IIF4 to a greater extent than with TEPC 183 (at 5.0 and 2.5 ug/ml, $p < 0.1$). The reactivity of collagen I with IIF4 was re-evaluated in competitive inhibition studies (Figure 8). These results indicated that a collagen compound contained the epitope reactive with IIF4; this epitope was not part of laminin or fibronectin.

Conformation of IIF4 Reactivity with Collagen IV

The experiments indicated that collagen IV contained the epitope recognized by IIF4; to confirm this result, IIF4 was competitively inhibited by isolated components of GBM (Figure 8).

A. IIF4 Reactivity is not Inhibited by Laminin, Fibronectin, Heparan Sulfate Proteoglycan, or Bovine Serum Albumin

IIF4 (2.5 ug/ml) was reacted with proteins (100 to 0.01 ug/ml). Unbound IIF4 antibody was assayed for by applying the IIF4 protein reaction mixture to trypsin digested SCM plates. The lower panel of Figure 8 represents IIF4 reactivity not inhibited by laminin, fibronectin, heparan sulfate proteoglycan and bovine serum albumin; none of these proteins was able to inhibit IIF4 reactivity to any extent distinguishable from no or very low levels of antibody inhibition. Bovine serum albumin was included as a globular protein

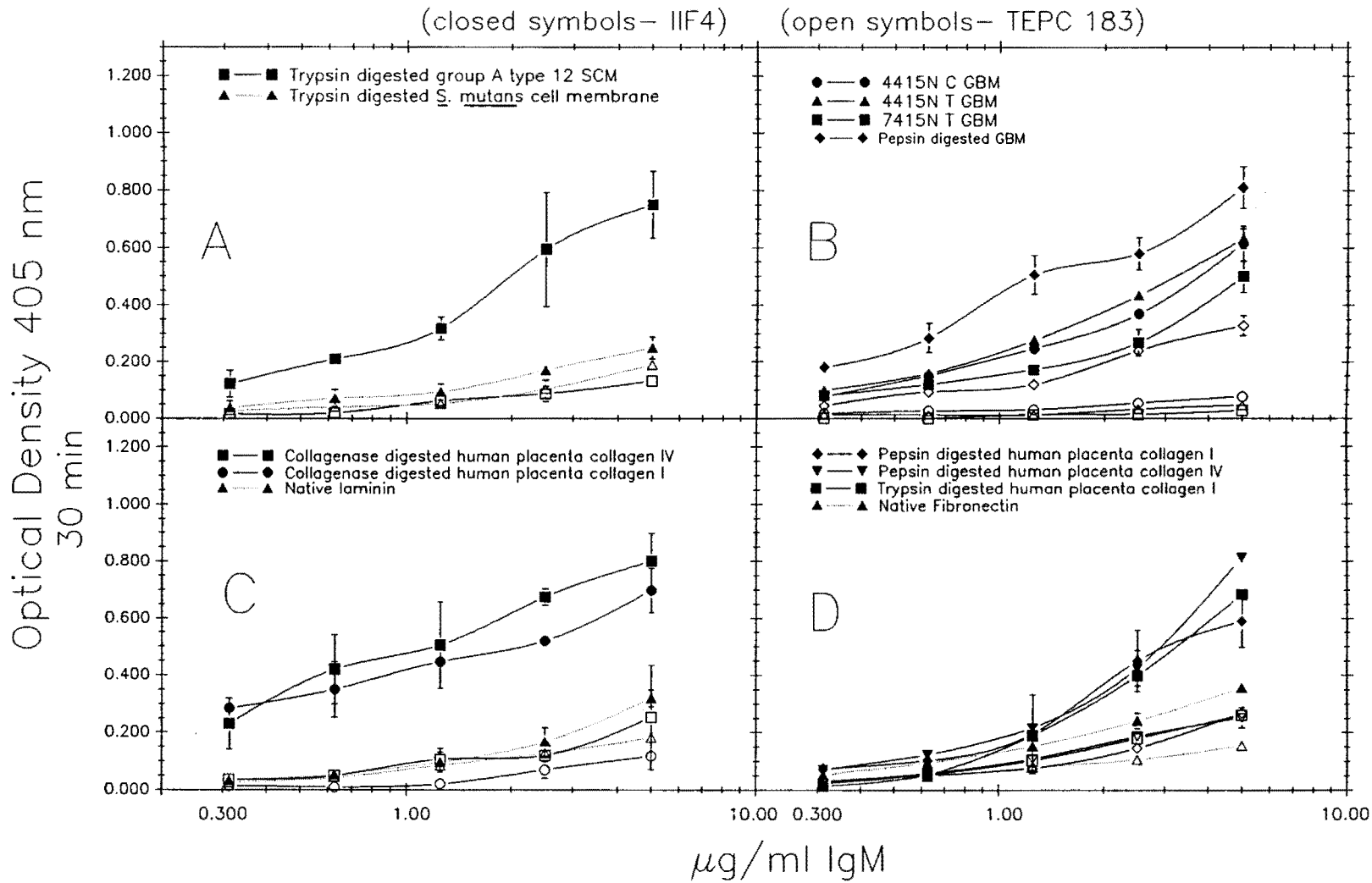
not part of the GBM. These results confirm the ELISA data previously presented; IIF4 did not react with GBM components fibronectin, laminin, heparan sulfate proteoglycan.

B. IIF4 Reactivity is Inhibited by Collagen IV

IIF4 was reacted with GBM and collagen preparations and unbound antibody was assayed as described. The upper panel of Figure 8 represents IIF4 reactivity inhibited by enzyme digested GBMs and enzyme digested collagen I and IV. Pepsin digested collagen I did not inhibit IIF4 reactivity with SCM to any extent distinguishable from no or very low levels of IIF4 reactivity inhibition. Trypsin digested, collagenase digested, or pepsin digested GBMs inhibited IIF4 reactivity to maxima of 52%, 40%, and 63%, respectively. These results confirmed the IIF4 reactivity with trypsin, collagenase, or pepsin digested GBMs as indicated in Figure 7. Results presented in Figure 8 also confirmed that collagen IV contained the epitope recognized by IIF4. IIF4 was inhibited to maxima of 73% and 43% by pepsin digested collagen IV and collagenase digested collagen IV, respectively. These results confirmed that collagen IV, and not other GBM components and not collagen I, contains the IIF4 reactive epitope. A level of 40% IIF4 inhibition was chosen to include the reproducible 40% inhibition of IIF4 reactivity by collagenase digested GBM. Amounts of GBM preparations and collagen IV preparations required to inhibit 40% of IIF4 reactivity are summarized in Table 3.

Figure 7. Comparison of IIF4 Reactivity with Streptococcal Cell Membranes, Isolated Components of GBM, and GBM

This figure is a compilation of IIF4 reactivity versus bacterial cell membrane components, isolated GBM components and enzyme digested GBMs. All assays were performed with 96 well plates coated with 0.5 ug/ml of the appropriate protein. After blocking, plates were treated with serial dilutions of IIF4 or TEPC 183 (10.0 to 0.05 ug/ml IgM in PBS). Plates were processed as described in METHODS AND MATERIALS. Each assay was performed a minimum of three times and the average reading and standard error is shown.



C. IIF4 Reactivity with Pepsin Digested Collagen IV is Inhibited by Trypsin Digested SCM and Pepsin Digested Collagen IV

Competitive inhibition experiments demonstrated that collagenase or pepsin digested collagen IV inhibited IIF4 reactivity with the IIF4 autologous antigen, SCM. Figure 9 represents experiments in which the ability of SCM to inhibit IIF4 reactivity with pepsin digested collagen IV was tested. Pepsin digested collagen IV inhibited IIF4 reactivity with pepsin digested collagen IV to a maximum of 58% while SCM inhibited IIF4 reactivity with pepsin digested collagen IV to a maximum of 42%. These results are compared for amount of inhibitor required to achieve 40% inhibition of IIF4 reactivity (summarized in Table 3). These results further show the IIF4 reactive epitope is located in collagen IV.

D. Summary of IIF4 Inhibition by Bacterial Membranes, GBMS, and GBM Components.

Table 3 represents the compilation of the IIF4 competitive inhibition studies presented in Figures 2, 8, and 9. The ability of trypsin digested SCM or *S. mutans* cell membrane to inhibit IIF4 reactivity with SCM to 40% was examined by first-order linear regression. It was found that 900 ng of SCM were required to achieve this level of IIF4 inhibition; *S. mutans* cell membrane was found to be non-inhibitory for IIF4.

Figure 8. Competitive Inhibition of IIF4 Reactivity with Isolated Proteins from the GBM

Competitive inhibition of IIF4 reactivity was assayed by testing the ability of an inhibitor to bind IIF4 and prevent IIF4 from binding to a trypsin digested SCM sensitized 96 well plate. The assay is described in METHODS and MATERIALS. As represented in the lower panel, fibronectin, bovine serum albumin heparan sulfate proteoglycan, and laminin are competitively reacted with IIF4. As represented in the upper panel, whole enzyme digested GBM preparations and enzyme digested collagens are competitively reacted with IIF4. These data are summarized in Table 3.

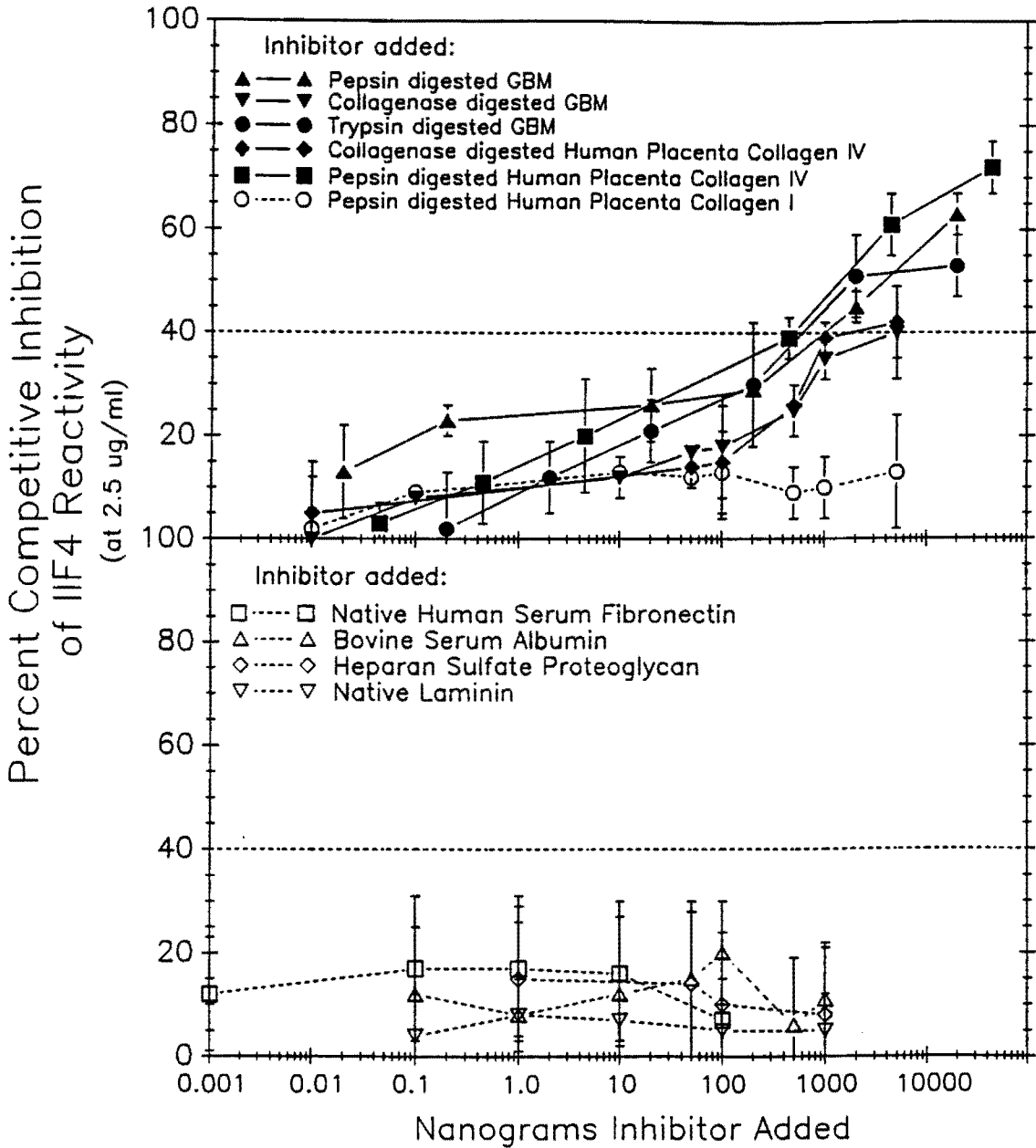
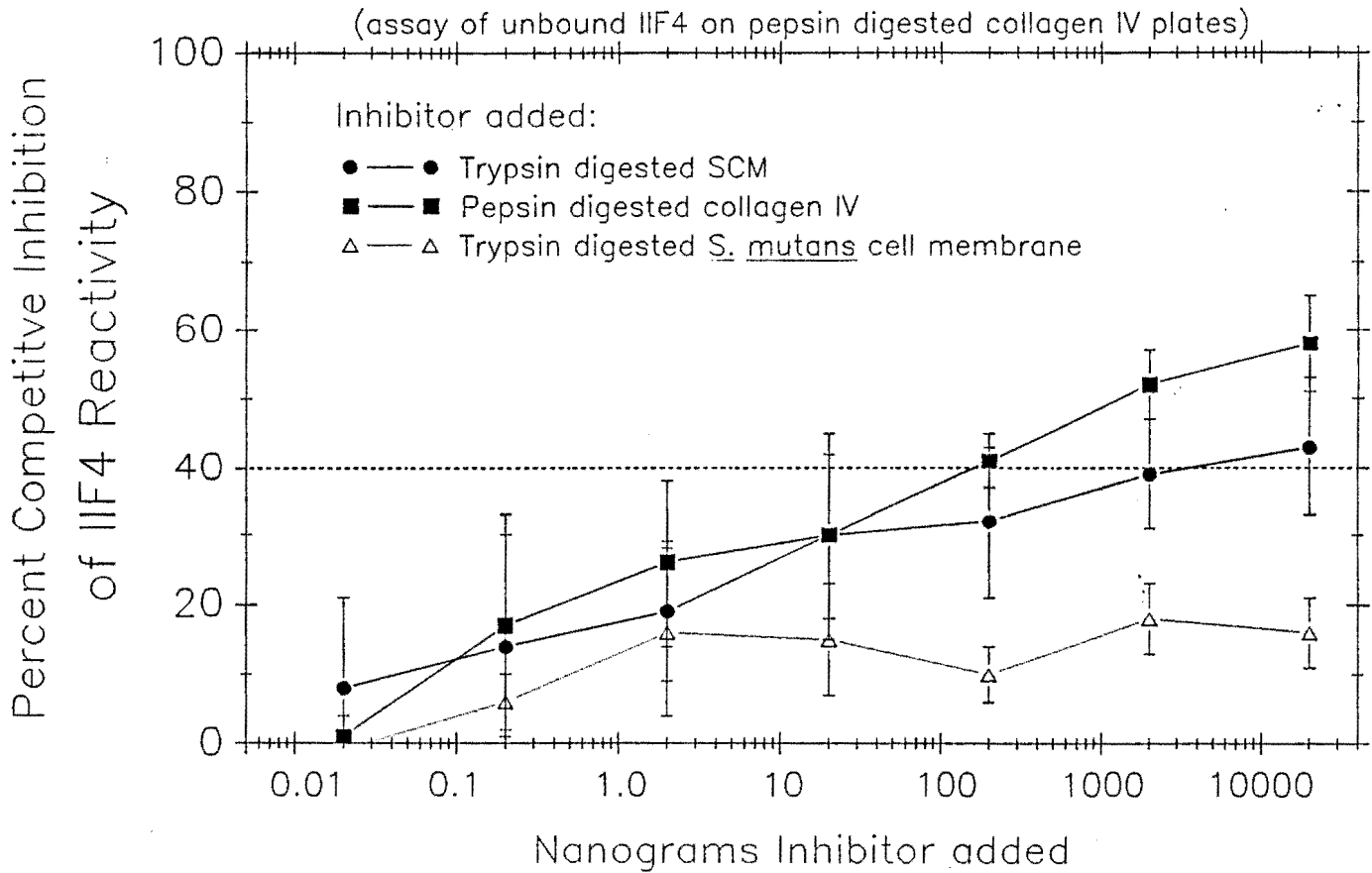


Figure 9. Competitive Inhibition of IIF4 Reactivity

Competitive inhibition of IIF4 reactivity was assayed by testing the ability of an inhibitor to bind IIF4 and prevent IIF4 from binding to a pepsin digested collagen sensitized 96 well plate as described in Materials and Methods. This experiment is the reciprocal of the experiment represented in Figure 8. These data are represented in Table 3.



Amounts of collagenase, trypsin, or pepsin digested GBMs required to achieve 40% inhibition of IIF4 reactivity were 900 ng, 500 ng, and 1000 ng, respectively. Approximately one-half the amount of trypsin digested GBM was required to inhibit IIF4 as compared to amounts required of collagenase or pepsin digested GBMs to inhibit IIF4. Approximately the same amount of SCM was required to inhibit IIF4 as was required of collagenase or pepsin digested GBMs.

Other components of the GBM, fibronectin, laminin, and heparan sulfate proteoglycan, were found to be non-reactive with IIF4. Bovine serum albumin was also non-inhibitory for IIF4. Contrary to data presented in Figure 7, pepsin digested collagen I was found to be non-inhibitory for IIF4 in these competitive inhibition studies.

Amounts of pepsin digested collagen IV and collagenase digested collagen IV required to achieve 40% inhibition of IIF4 reactivity with SCM were 300 ng and 1050 ng, respectively. The amount of pepsin digested collagen IV required to achieve 40% inhibition of IIF4 was one-third the amounts required of SCM, collagenase digested GBM and pepsin digested GBM.

The ability of SCM, S. mutans cell membrane, and pepsin digested collagen IV to inhibit IIF4 reactivity with pepsin digested collagen IV was also compared for 40% inhibition of IIF4 reactivity and are summarized in Table 3. S. mutans cell membrane was found to be non-reactive with IIF4 while 2000 ng and 200 ng of SCM and pepsin digested collagen IV, respectively, were required to achieve 40%

inhibition of IIF4 reactivity. These values are approximately the same as required of these inhibitors to inhibit IIF4 reactivity with SCM. These results confirm the presence of the IIF4 epitope in collagen IV. Pepsin digested collagen IV was the best inhibitor of IIF4 reactivity with SCM.

IIF4 Reactive Polypeptides in Collagen IV and GBM

Competitive inhibition studies established collagen IV as the GBM component containing the IIF4 recognized epitope. Collagen IV is a complex molecule consisting of three chains (any combination of alpha 1 chains and alpha 2 chains). IIF4 reacted with collagen IV and it was hypothesized that the location of the IIF4 reactive epitope on collagen IV could be identified. Immunoprecipitation of collagen IV and GBM polypeptides from enzyme digests were used to test this hypothesis.

A. Immunoprecipitation of GBM Polypeptides with IIF4

In order to demonstrate GBM polypeptide reactive with IIF4, immunoprecipitation with IIF4 was performed with eight GBMs. IIF4 immunoprecipitated polypeptides of 120, 100, and 36 kDa from collagenase digested GBM 11741 N (Figure 10, lane 3). No GBM polypeptides were precipitated with TEPC 183 (lane 5). In collagenase digested GBM 7415 N, only the 120 kDa polypeptide was IIF4 immunoprecipitated (Figure 11, A) and not with TEPC 183. In collagenase digested GBM 19763 N, IIF4 did immunoprecipitated the

TABLE 3.

SUMMARY OF COMPETITIVE INHIBITION STUDIES
Inhibition of IIF4 Reactivity

Inhibitor	Nanogram amount of inhibitor required to achieve 40% inhibition of IIF4 reactivity (ON A TRYPSIN DIGESTED SCM PLATE) (at 250 ng of IIF4)
Trypsin digested SCM	900 +/- 250 ng
Trypsin digested <u>S. mutans</u> cell membrane	NI
Collagenase digested GBM	900 +/- 300 ng
Trypsin digested GBM	500 +/- 95 ng
Pepsin digested GBM	1000 +/- 300 ng
Bovine serum albumin	NI
Human serum fibronectin	NI
Murine laminin	NI
Bovine kidney heparan sulfate proteoglycan	NI
Pepsin digested human placenta collagen I	NI
Pepsin digested human placenta collagen IV	300 +/- 150 ng
Collagenase digested human placenta collagen IV	1050 +/- 360 ng

	Nanogram amount of inhibitor required to achieve 40% inhibition of IIF4 reactivity (at 250 ng of IIF4) (ON A PEPSIN DIGESTED COLLAGEN IV PLATE)
Trypsin digested SCM	2000 +/- 700 ng
Trypsin digested <u>S. mutans</u> cell membrane	NI
Pepsin digested collagen IV	200 +/- 100 ng

NI-- not inhibitory at 40% inhibition of IIF4 reactivity.
Nanogram inhibitory values were determined from a first-
order linear regression plot of each inhibitor and at 40%
inhibition of IIF4 reactivity.

polypeptides of 120, 100, and 39 kDa; TEPC 183 did not immunoprecipitate any apparent GBM polypeptides (Figure 11, B). In two of the GBMs, the 120 and 100 kDa polypeptides were IIF4 immunoprecipitated [collagenase digested GBMs: 11778 N (Figure 12) and 9662 N (Figure 13) (Table 4)]. These experiments indicated that IIF4 reacted with higher molecular weight collagenase digestion products of GBM (120 and/or 100 kDa) and sometimes with a 36-39 kDa GBM polypeptide.

In Figure 14, collagenase digested GBM, 8961 N, was immunoprecipitated with IIF4. A 120, 100, and 26 kDa GBM polypeptide with IIF4 immunoprecipitated (lanes 3 and 4; lane 4 was a reaction of one half the amount of GBM in the immunoprecipitation). This reaction appeared to be specific since preclearing of the collagenase digested GBM with IIF4 prior to immunoprecipitation with IIF4 removed the 120, 100, and 26 kDa GBM polypeptides from the reaction mixture (lane 5). TEPC 183 did not precipitate any GBM polypeptides and preclearing of the GBM preparation with TEPC 183 did not have any effect on the elution profile (lanes 6 and 7, respectively).

For comparison, a pepsin digest of GBM was made and immunoprecipitated by IIF4. IIF4 immunoprecipitated two polypeptides from a pepsin digested GBM which were 100 and 85 kDa (Figure 15, lane 3). IIF4 precleared these polypeptides (lane 4) and TEPC 183 did not immunoprecipitate any polypeptides of pepsin digested GBM. These results are summarized in Table 4.

Figure 10. Immunoprecipitation of Collagenase Digested GBM Polypeptides with IIF4

IIF4 bound to goat anti-mouse agarose beads was reacted with 100 ug of collagenase digested GBM 11741 N and processed as described in METHODS AND MATERIALS. Lane 2 represents the separation profile of collagenase digested GBM on 15% SDS-PAGE. Lane 3 is the profile of IIF4 immunoprecipitated GBM. Lane 5 represents TEPC 183 precipitated GBM polypeptides. Lane 4 represents IIF4 immunoprecipitated alone. The gamma, mu, light, and J chains are as labeled.

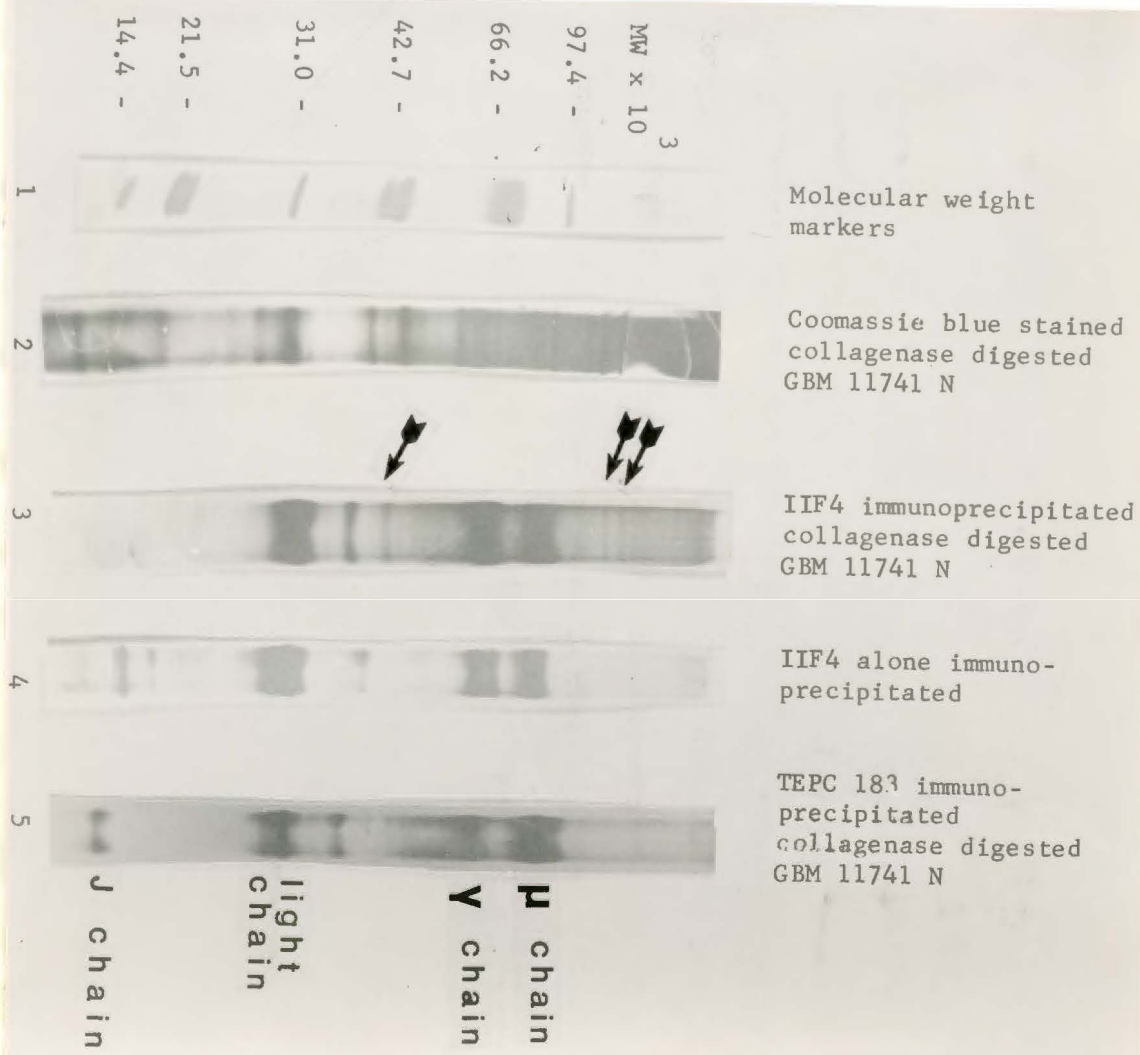


Figure 11. Immunoprecipitation of Polypeptides from Collagenase
Digested GBMs 7415 N and 19763 N

Collagenase digested GBMs 7415 N and 19763 N were processed as described. In panel A, IIF4 immunoprecipitated GBM 7415 N is represented Lane 4 represents TEPC 183 immunoprecipitated GBM 7415 N. In panel B, IIF4 immunoprecipitated GBM 19763 N is represented (lane 7). TEPC 183 precipitated GBM 19763 N is represented in lane 8.

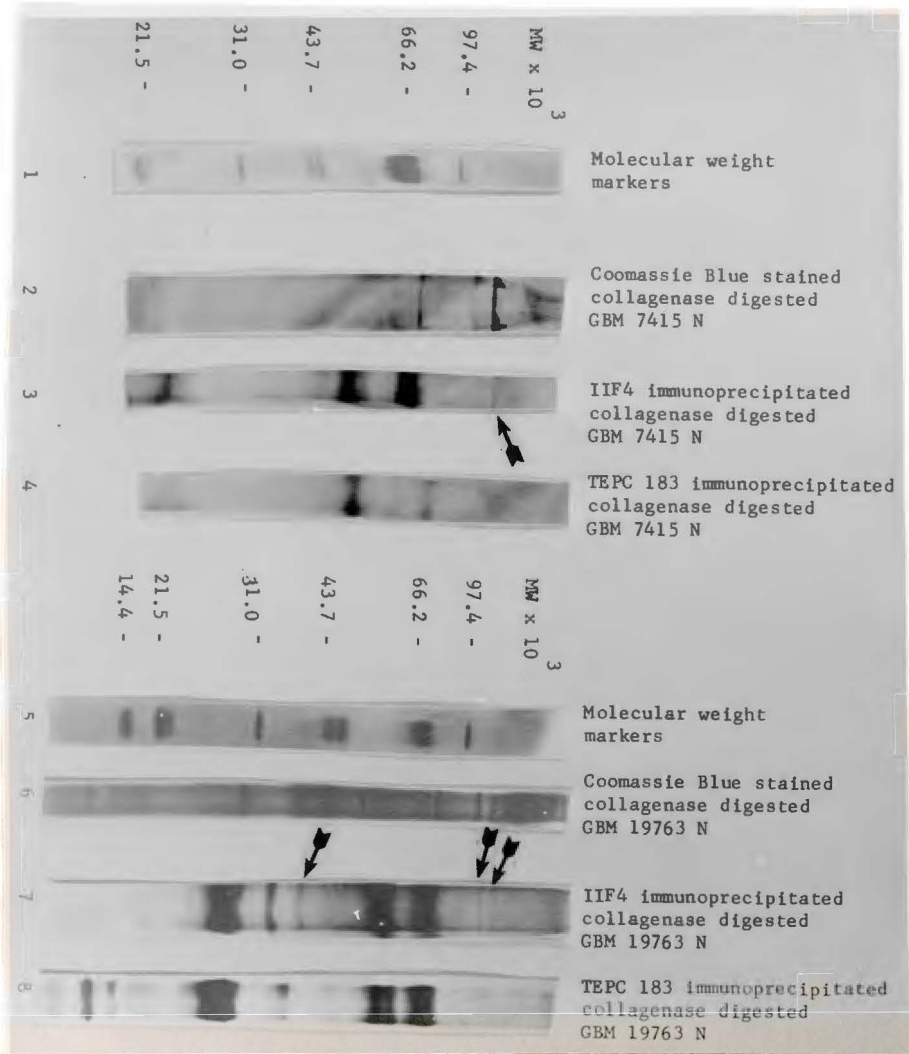


Figure 12. Immunoprecipitation by IIF4 of Polypeptides from Collagenase Digested GBM 11778 N

Collagenase digested GBM was processed as described in Figure 10. IIF4 immunoprecipitated collagenase digested GBM 11778 N (lane 3) and TEPC 183 immunoprecipitated GBM (lane 4) are represented. IIF4 immunoprecipitated alone (lane 5), TEPC 183 immunoprecipitated alone (lane 6), and goat anti-mouse IgM agarose beads processed alone (lane 7) are included as antibody chain mobility references.

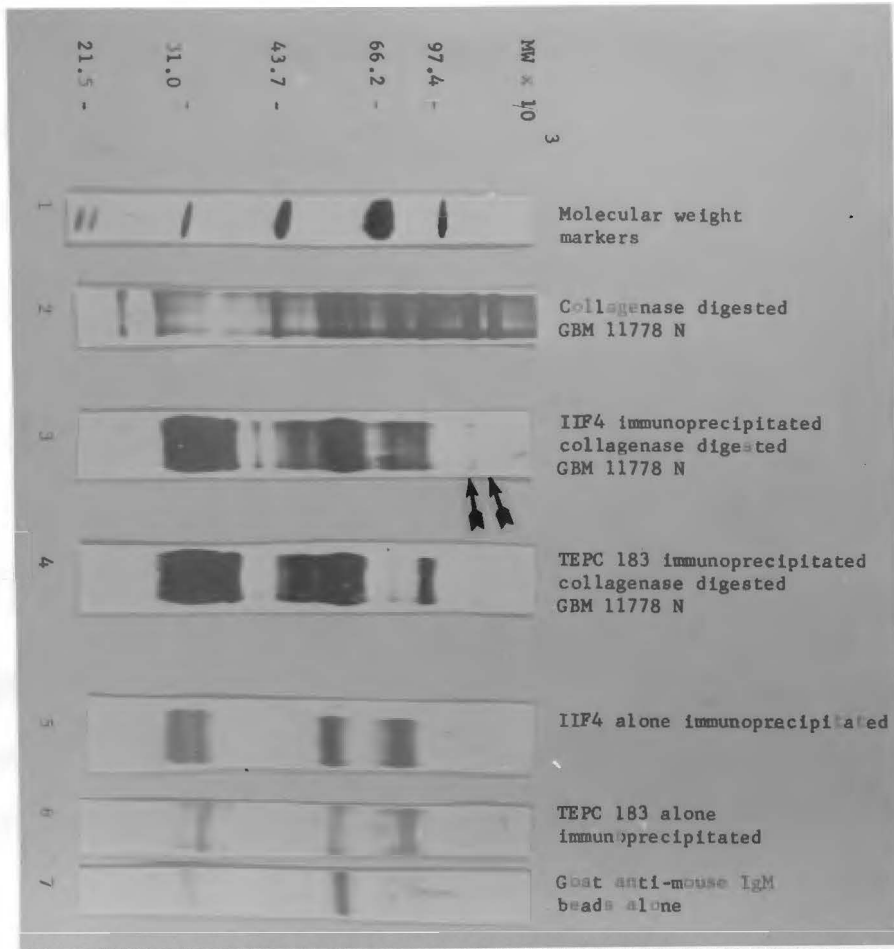


Figure 13. Immunoprecipitation by IIF4 of Polypeptides from Collagenase Digested GBM 9662 N

Collagenase digested GBM was processed as described in Figure 10. IIF4 immunoprecipitated collagenase digested GBM 9662 N in lane 3 and TEPC 183 immunoprecipitated GBM polypeptides (lane 4) are represented. IIF4 immunoprecipitated alone (lane 5), TEPC 183 immunoprecipitated alone (lane 6), and goat anti-mouse IgM agarose beads processed alone (lane 7) are included as antibody chain mobility references.

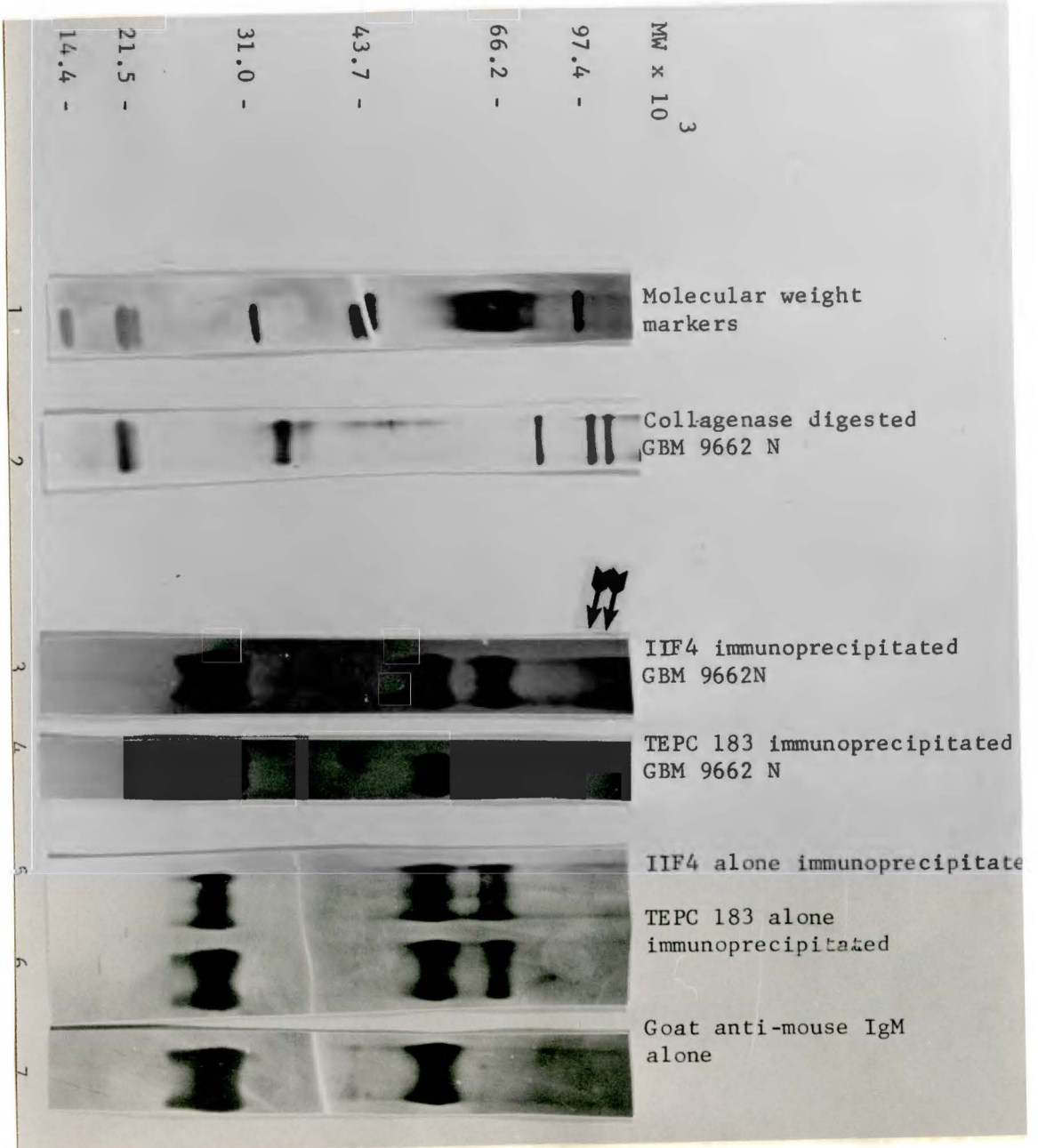


Figure 14. Immunoprecipitation of Polypeptides from Collagenase
Digested GBM 8961 N

Collagenase digested GBM (100 ug) was IIF4 immunoprecipitated (lane 3). Collagenase digested GBM (50 ug) was IIF4 immunoprecipitated (lane 4). IIF4 precleared GBM digest is represented in lane 5. TEPC 183 immunoprecipitated and TEPC 183 precleared of the GBM are represented (lanes 6 and 7, respectively). IIF4 immunoprecipitated alone (lane 8), TEPC 183 immunoprecipitated alone (lane 9), and goat anti-mouse IgM agarose beads processed alone (lane 10) are included as antibody chain mobility references.

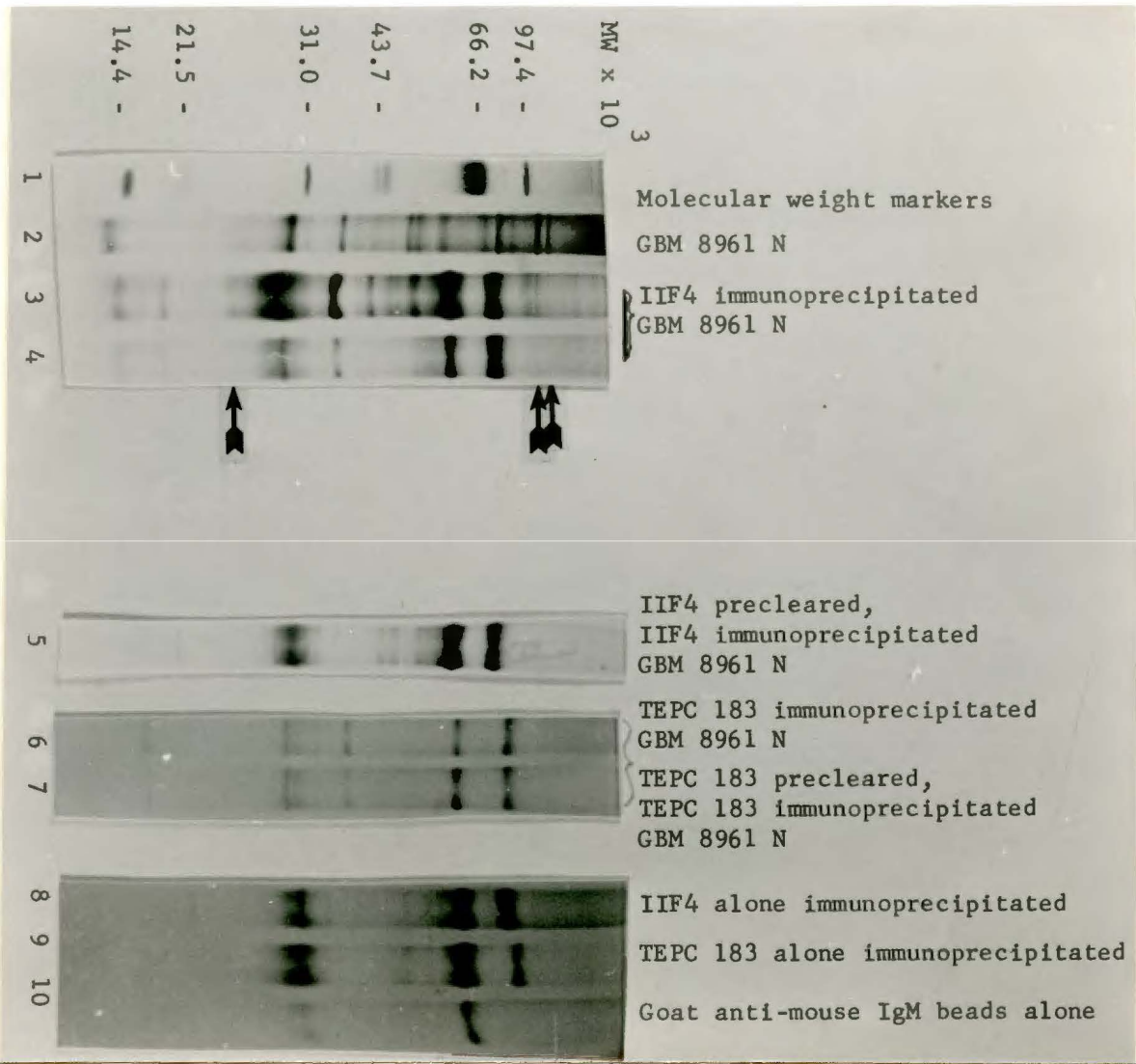
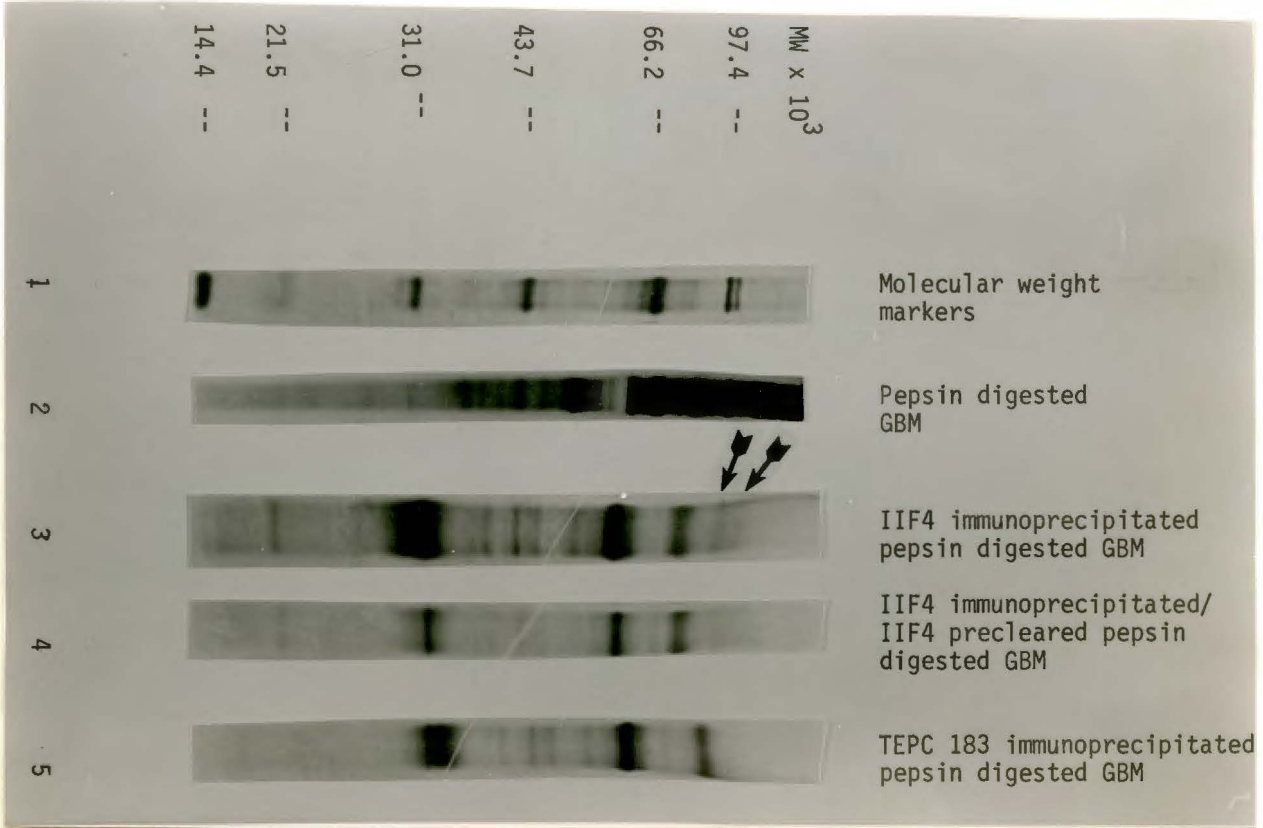


Figure 15. Immunoprecipitation of Pepsin Digested GBM with IIF4

Separation of pepsin digested GBM polypeptides is represented in lane 1. IIF4 immunoprecipitated pepsin digested GBM is represented in lane 3. IIF4 precleared GBM is represented (lane 4). TEPC 183 precipitated pepsin digested GBM polypeptides is represented (lane 5).



B. Polypeptides from Collagenase Digested Collagen IV Reactive with IIF4

Collagenase digested collagen IV was subjected to immunoprecipitation with IIF4 or TEPC 183 (Figure 16). Collagen IV polypeptides of 120 and 100 kDa were immunoprecipitated by IIF4.

C. Polypeptides from Pepsin Digested Collagen I and Collagen IV Reactive with IIF4

IIF4 immunoprecipitated polypeptides of 200, 140, and 90 kDa pepsin digested collagen IV (Figure 17A, lane 2) and these polypeptides were precleared by IIF4 (lane 3). This indicated that these polypeptides were specific for IIF4. TEPC 183 did not precipitate these polypeptides (lane 4). Preclearing the pepsin digested collagen IV with TEPC 183 demonstrated higher molecular weight polypeptides (200, 140, and 90 kDa) which corresponded to the polypeptides IIF4 immunoprecipitated with IIF4 (lane 2). Since TEPC 183 had not immunoprecipitated these polypeptides (lane 4); this result is puzzling and it may not be concluded that IIF4 specifically immunoprecipitated these polypeptides. Laminin was not immunoprecipitated by either IIF4 or TEPC 183 (Figure 17B, lanes 6-10).

For comparison, pepsin digested collagen I was immunoprecipitated by IIF4. Neither IIF4 nor TEPC 183 immunoprecipitated any pepsin digested collagen I polypeptides (Figure 17B, lanes 2, 4 and 7, 9 respectively) and preclearing pepsin digested collagen I with either IIF4 or TEPC 183 had no

effect on the elution profile (lanes 3 and 5, respectively). These results are summarized in Table 4.

Amino Acid Analysis of IIF4 Reactive Polypeptides

In order to characterize IIF4 reactive polypeptides from GBM and equivalently sized polypeptides from collagen IV, collagenase and pepsin digested collagen IV and GBM (19474N, 19763N) were separated on 6.5% SDS-PAGE. The 120 and 100 kDa polypeptides were recovered from the collagenase digests of the GBMs and from collagen IV; the 100 kDa polypeptides were recovered from the pepsin digestions of the GBM and collagen IV. All recovered polypeptides were subjected to amino acid analysis (Figures 18 and 19). The IIF4 reactive polypeptides from collagenase digested GBM or collagen IV (120 and 100 kDa) were similar in their content of aspartic acid (10%), glutamic acid (11%), leucine (8%), and lysine (8%). Each polypeptide had collagenous character: glycine (10%), hydroxyproline and hydroxylysine (1%). Of particular note is the cysteine residue content of 2-4%. The 100 kDa polypeptides from pepsin digested collagen IV or GBM were similar in their content of aspartic acid (8%), serine (8%), glutamic acid (4%) and leucine (7%). Each polypeptide had collagenous character: glycine (17%), hydroxylysine and hydroxyproline (1-3%).

Figure 16. IIF4 Immunoprecipitated Polypeptides from Collagenase
Digested Collagen IV.

Lane 2 represents IIF4 immunoprecipitated collagenase digested collagen IV. Lane 3 represents TEPC 183 immunoprecipitated collagenase digested collagen IV. IIF4 immunoprecipitated alone (lane 4), TEPC 183 immunoprecipitated alone (lane 5) are included as antibody chain mobility references.

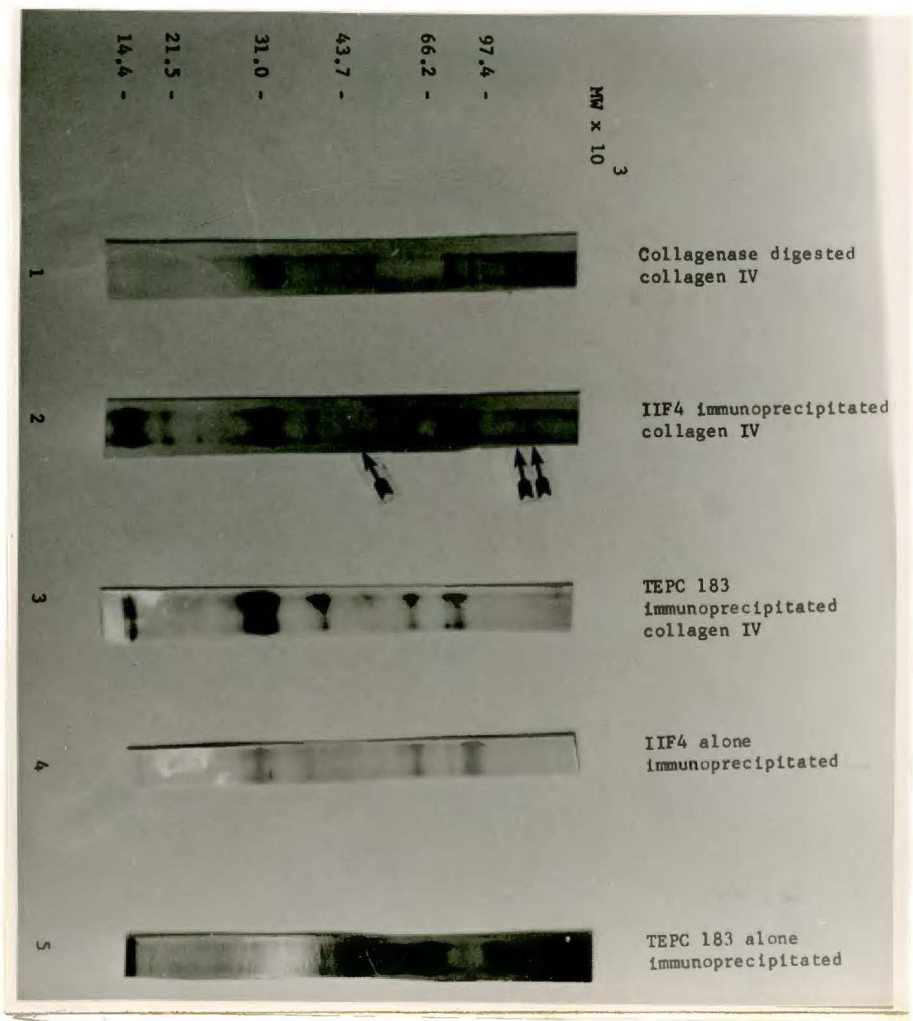


Figure 17A. IIF4 Immunoprecipitation of Pepsin Digested Collagen IV

The IIF4 immunoprecipitation of pepsin digested collagen IV is represented. IIF4 immunoprecipitated pepsin digested collagen IV is represented in lane 2. IIF4 precleared collagen IV is represented in lane 3. TEPC 183 immunoprecipitated and TEPC 183 precleared collagen IV are represented in lanes 4 and 5, respectively. IIF4 immunoprecipitated alone (lane 6), TEPC 183 immunoprecipitated alone (lane 7), and goat anti-mouse IgM agarose beads processed alone (lane 8) are included as antibody chain mobility references. The data represented in Figure 17B was part of the experiment shown here.

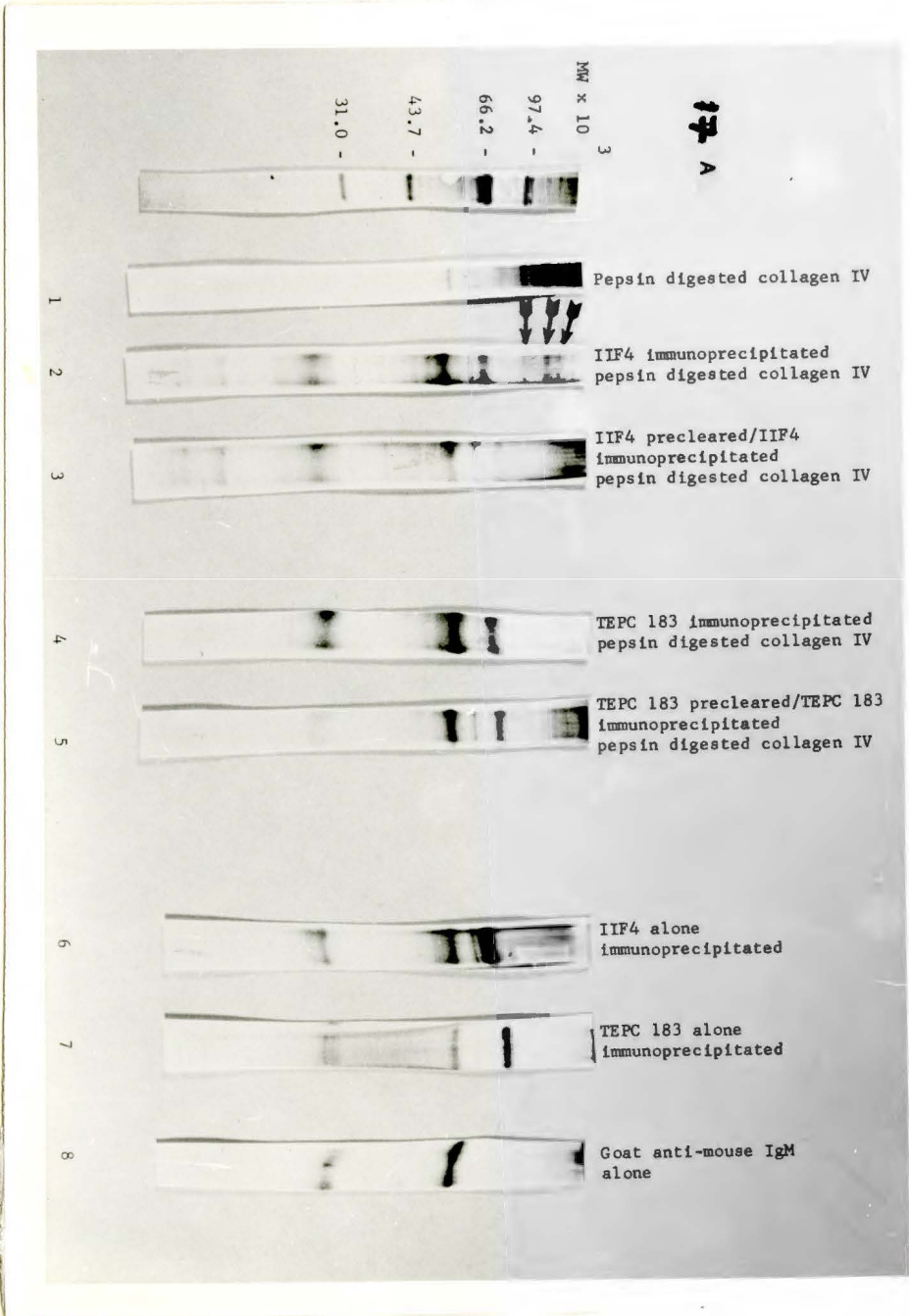


Figure 17B. IIF4 did not Immunoprecipitate any Polypeptides from Pepsin Digested Collagen I or Laminin

These data are part of the experiment shown in Figure 17A. IIF4 and TEPC 183 immunoprecipitated or precleared pepsin digested collagen I are represented (lanes 2, 3, 4, and 5). IIF4 or TEPC 183 immunoprecipitated or precleared laminin is represented (lanes 7, 8, 9, 10). IIF4 immunoprecipitated alone (lane 11), TEPC 183 immunoprecipitated alone (lane 12) and the goat anti-mouse agarose beads processed alone (lane 13) are included for antibody chain mobility references.

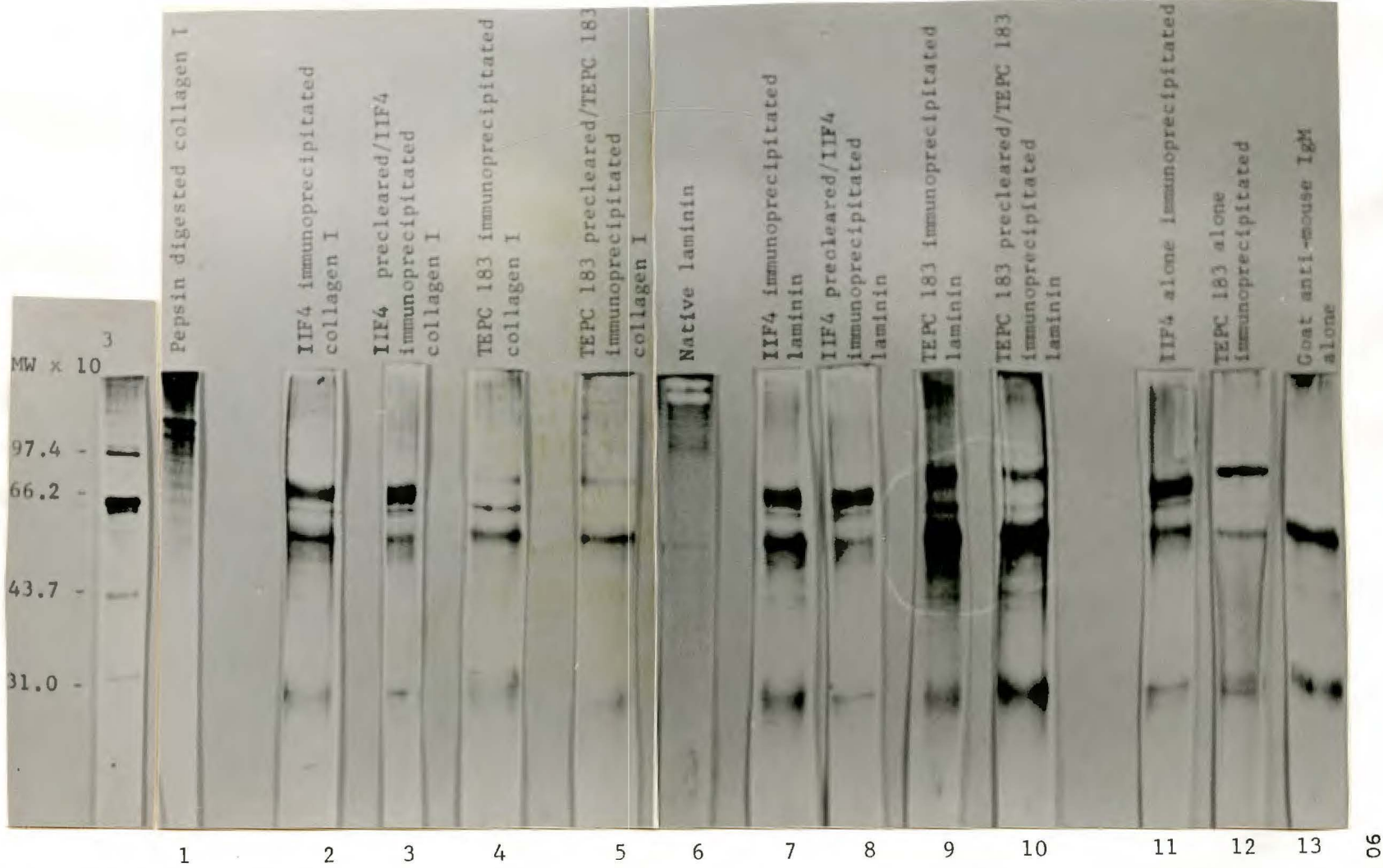


TABLE 4.

SUMMARY OF IMMUNOPRECIPITATION DATA

	kDa polypeptide immunoprecipitated with IIF4		
<hr/>			
Collagenase digested GBMs:			
11741 N	120, 100,		37
11778 N	140, 120, 100		
9662 N	120, 100		
7415 N	120		
19763 N	120, 100,		40
8961 N	120, 100,		27
Pepsin digested GBM ¹	100, 85		
<hr/>			
Pepsin digested collagen IV ²	200, 140,	90	
Pepsin digested collagen I	none		
<hr/>			
Collagenase digested collagen IV	120, 100,		45
Laminin	none		
<hr/>			

¹ Two aliquots of GBM (18274) were pepsin digested separately and subjected to IIF4 or TEPC 183 immunoprecipitation. The data presented here and in Figure 17A is a compilation of these two digestions and immunoprecipitations.

² IIF4 alone did not immunoprecipitate pepsin digested collagen IV polypeptides; TEPC 183 also had reactivity with these polypeptides (Figure 17A). The data is included here for comparison and completeness.

Anti-collagen IV mAb Reactivity with GBM

As seen with IIF4, anti-collagen IV mAb reacted with the GBM in human cortical tissue (Figure 20). Anti-collagen IV mAb reacted with the GBM in a linear deposition pattern as well as reacting with mesangial matrix, tubular basement membrane (not shown in photograph) and the Bowman's capsule. MOPC 21 did not react with the cortical tissue; yellow-orange autofluorescence of cortical nuclei was seen in MOPC 21 staining as it had been in TEPC 183 staining (Figure 3B). These results demonstrated that anti-collagen IV mAb reacted with tissue components known to contain collagen IV.

Comparison of Anti-Collagen IV mAb Reactivity among Bacterial Cell Membranes, Isolated Proteins Comprising the GBM, and GBMs

Since, as represented in Figures 7, 8, and 9, IIF4 reacted with collagen proteins and not laminin or fibronectin, and since collagen IV comprises up to 80% of the GBM (Wieslander, 1983), the reactivity of the bacterial cell membranes and GBM preparations with anti-collagen IV mAb was investigated. This was done to contrast anti-collagen IV mAb reactivity with IIF4 reactivity among GBM components.

Trypsin digested SCM and trypsin digested cell membrane from S. mutans were reacted with anti-collagen IV mAb and MOPC 21 as a control (20 to 2.0 ug/ml IgG1). Both bacterial cell membrane preparations did not react above background levels of MOPC 21 reactivity with anti-collagen IV mAb (Figure 21, A).

Figure 18. Amino acid profile of polypeptides from collagenase digested GBM and collagen IV

The amino acid quantity from each eluted polypeptide is shown here as an average of two determinations for that polypeptide. All collagenase digests of the 120 and 100 kDa GBM and collagen IV polypeptides had similar amounts of cysteine, aspartic acid, threonine, serine, glutamic acid, alanine, isoleucine, leucine, tyrosine, lysine, and arginine. All digests contained small amounts of hydroxyproline and hydroxylysine.

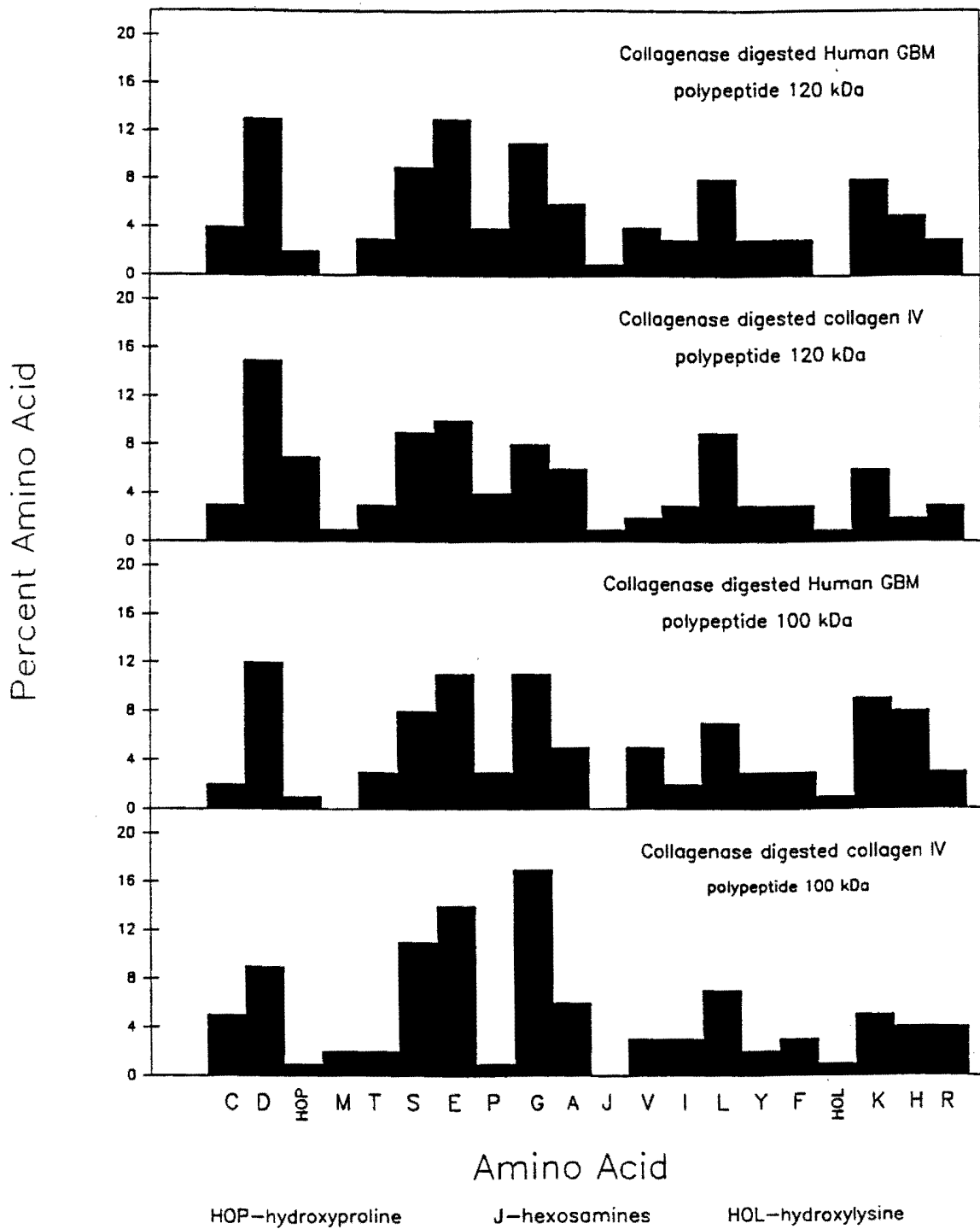


Figure 19. Amino acid profile of polypeptides from pepsin digested GBM and collagen IV

Isolated polypeptides from pepsin digested GBM or collagen IV of 100 kDa had very similar amounts of most amino acids. Each polypeptide contained an equivalent amount each of cysteine, hydroxyproline, hydroxylysine, and glycine.

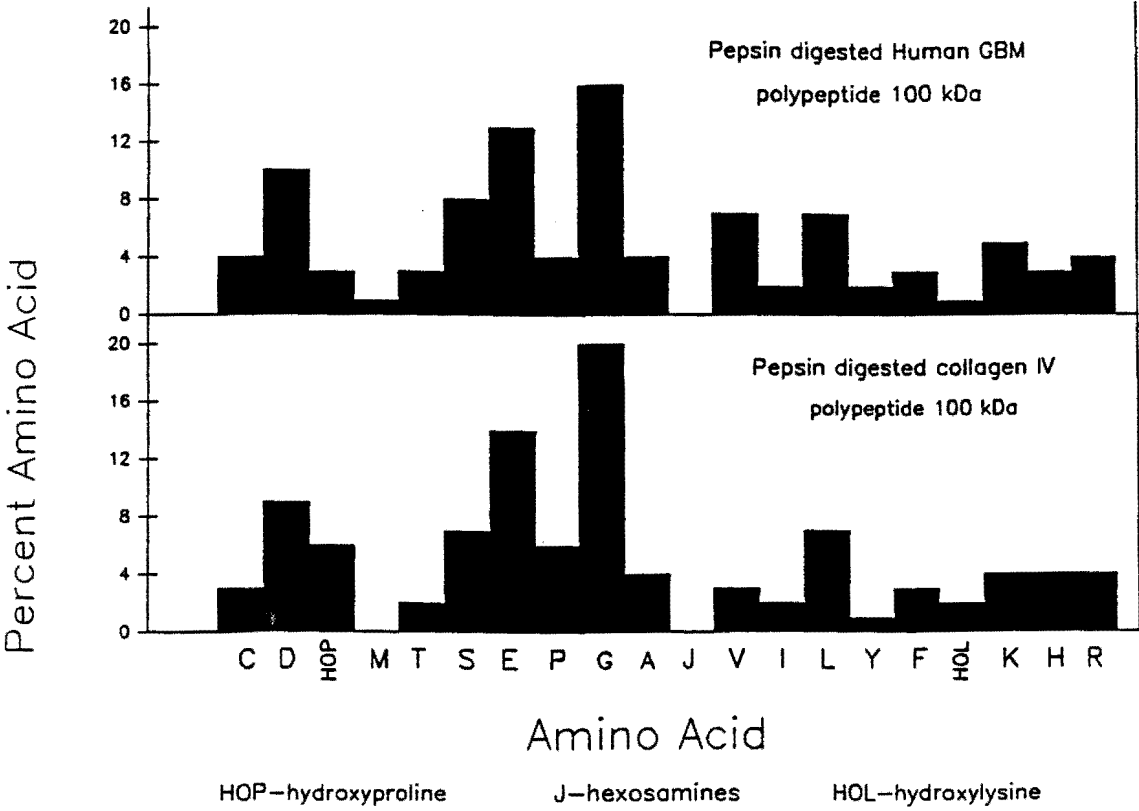
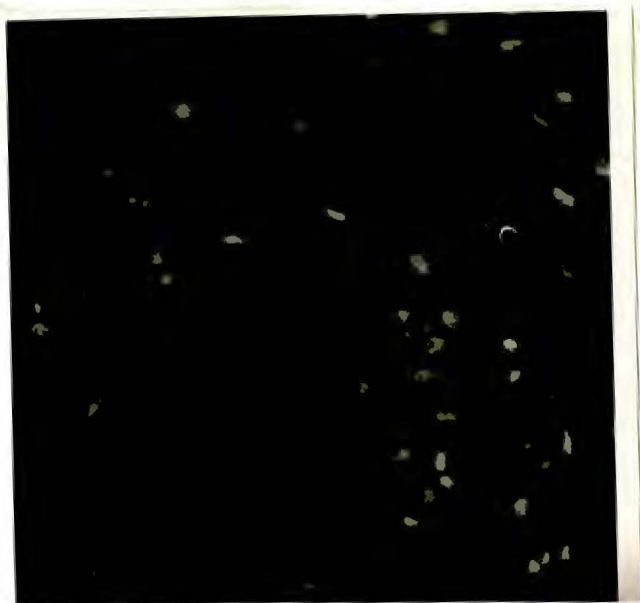
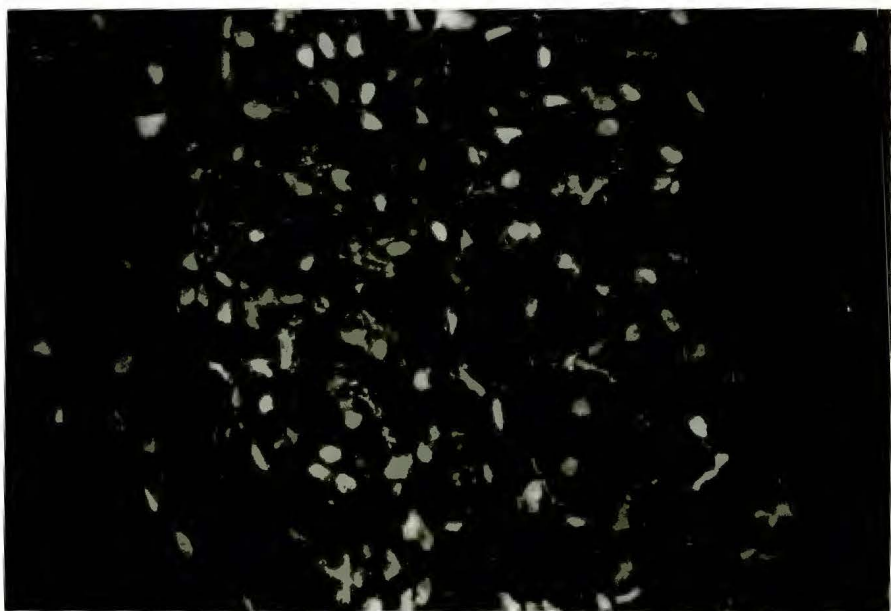


Figure 20. Indirect Immunofluorescence of Human Renal Cortical Tissue with Anti-Collagen IV mAb

Cryostat sectioned human renal cortical tissue was reacted with 10 ug of anti-collagen IV mAb or MOPC 21. After processing, sections were viewed under a fluorescent microscope. In A, reactivity of human cortical tissue with anti-collagen IV mAb is shown. Bowman's capsule, the GBM and mesangial matrix were reactive with anti-collagen IV mAb. In B, MOPC 21 did not react with any portion of the cortical tissue. Mesangial nuclei were autofluorescent.



B



A

Reactions of anti-collagen IV mAb and enzyme digested GBMs are represented (panel B). Only pepsin and trypsin digested GBMs reacted with anti-collagen IV mAb. There was a distinct difference in the reactivity of anti-collagen IV mAb with the two trypsin digested GBMs shown: at 4 ug/ml anti-collagen IV mAb, trypsin digested GBM 4415 N reacted to a significantly lesser extent with anti-collagen IV mAb than with GBM 7415 N ($p < 0.001$), but the reactivity of GBM 4415 N with anti-collagen IV mAb was significantly greater than the reactivity of GBM 4415 N with MOPC 21 ($p < 0.01$). Only trypsin and pepsin digested GBMs contained the anti-collagen IV mAb epitope.

The reactivity of anti-collagen IV mAb with collagenase digested collagen I or collagen IV and with laminin is represented in Panel C. Laminin did not react above background levels of MOPC 21 reactivity with as much as 20 ug/ml IgG1 of anti-collagen IV mAb. Collagenase digested collagen I or collagen IV also did not react with as much as 20 ug/ml of anti-collagen IV mAb and this very low level of reactivity was indistinguishable from reactivity with MOPC 21 (in both cases, $p > 0.2$). This result is consistent with the data presented in Panel B. Pepsin or trypsin digested collagen I or collagen IV reacted with anti-collagen IV mAb (Panel D).

Trypsin or pepsin digested GBM or collagen reacted with anti-collagen IV mAb to significantly greater degrees than with MOPC 21 (in all cases and for all IgG1 concentrations, $p < 0.001$). Collagenase digested GBM or collagen could not react with anti-collagen IV mAb. These data indicated that the anti-collagen IV mAb

recognized epitope is destroyed by collagenase treatment and this implied that the anti-collagen IV mAb recognized epitope is located in the triple helical domain of collagen.

Fibronectin reacted with anti-collagen IV mAb (panel D). This result will be re-evaluated in COMPETITIVE INHIBITION OF ANTI-COLLAGEN IV mAb REACTIVITY.

Anti-Collagen IV mAb Reactivity with Trypsin or Collagenase Digested GBM

As seen in Figure 21, two collagenase digested GBMs and collagenase digested collagen I or IV did not react with anti-collagen IV mAb. As represented in Figure 22, none of the collagenase digested GBMs reacted with anti-collagen IV mAb to any significant extent while all trypsin digested GBM could react with anti-collagen IV mAb. The collagenase digested GBM most reactive was not significantly greater in reactivity with anti-collagen IV mAb than any other collagenase digested GBM (at 5.0 and 2.5 ug/ml IgG1, $p > 0.1$).

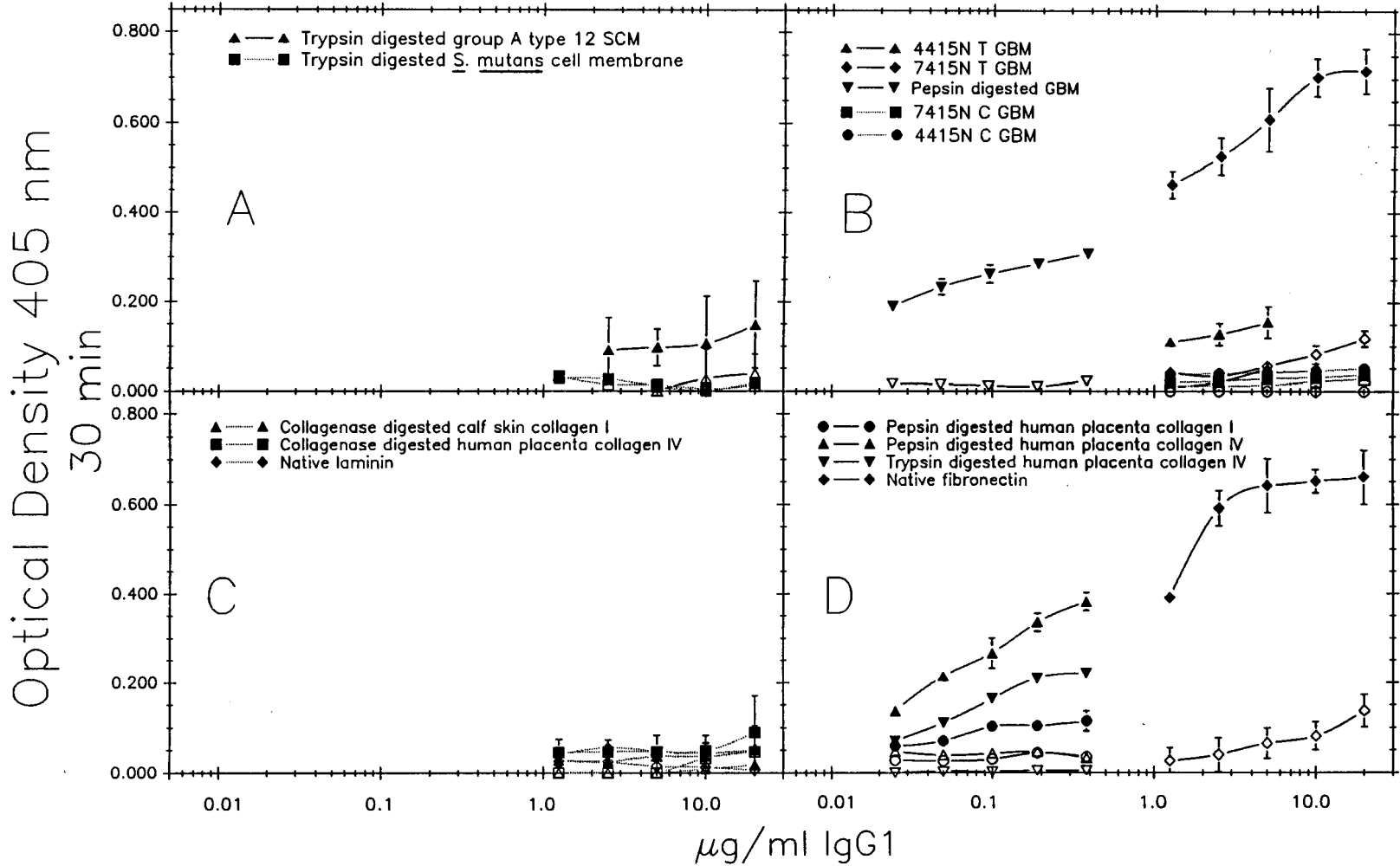
Competitive Inhibition of Anti-Collagen IV mAb Reactivity

For a comparison of inhibition of IIF4 reactivity with GBM or GBM components, competitive inhibition of anti-collagen IV mAb reactivity was performed. Both pepsin digested collagen I and IV inhibited anti-collagen IV mAb reactivity to maxima of 88 and 76%, respectively at 10,000 ng of collagen I or collagen IV. Neither

Figure 21. Anti-Collagen IV Reactivity with Bacterial Membranes, Isolated Components of the GBM, and GBMs

Anti-collagen IV mAb reactivity with bacterial cell membranes, isolated GBM components, and enzyme digested GBMs was assayed in ELISA. Reactivity of anti-collagen IV mAb with trypsin digested SCM and S. mutans cell membrane is represented in Panel A. Pepsin or trypsin digested collagen I and collagen IV reactions with anti-collagen IV mAb or MOPC 21 are represented in Panel B. Panel C represents anti-collagen IV mAb or MOPC 21 reactivity with laminin and collagenase digests of collagen I or IV. Panel D represents anti-collagen IV mAb and MOPC 21 reactivity with pepsin or trypsin digested collagen IV, pepsin digested collagen I and fibronectin.

(closed symbols: anti-collagen IV mAb) (open symbols: MOPC 21)



trypsin digested SCM nor native fibronectin inhibited anti-collagen IV mAb reactivity in this assay (Figure 23). The results of these assays are summarized in Table 5.

Summary of Inhibition of Anti-Collagen IV mAb Reactivity

Neither trypsin digested SCM nor native fibronectin inhibited anti-collagen IV mAb reactivity with trypsin digested GBM. Both pepsin digested collagen I and collagen IV inhibited anti-collagen IV mAb. Approximately twice as much collagen IV as collagen I was required to inhibit 40% of anti-collagen IV mAb reactivity. Since anti-collagen IV mAb reacted with both types of collagen, this result indicated that the anti-collagen IV mAb recognized epitope is located in domains shared by collagen IV and collagen I. This is the triple helical domain.

Figure 22. Trypsin Digested GBM and Collagenase Digested GBM Did Not React Equally with Anti-Collagen IV mAb

The reactivity of anti-collagen IV mAb and MOPC 21 with 7 trypsin digested GBMs and 12 collagenase digested GBMs is represented.

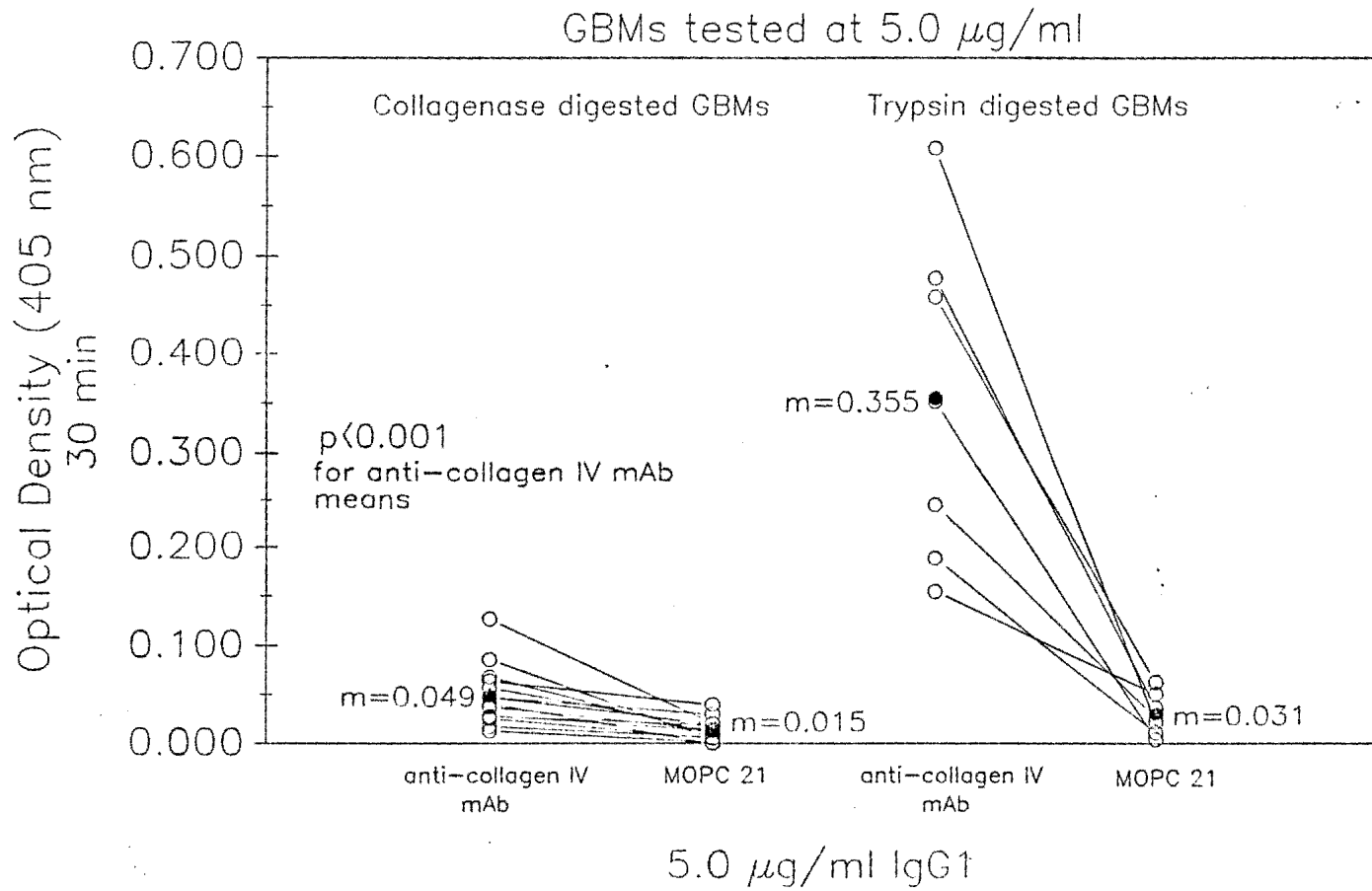


Figure 23. Competitive Inhibition of Anti-Collagen IV mAb Reactivity with Collagens I and IV, Fibronectin, and Trypsin Digested SCM

Competitive inhibition of anti-collagen IV mAb reactivity was assayed as described for IIF4 reactivity in Figure 13 except that 0.5 ug/ml anti-collagen IV mAb was used and inhibition of anti-collagen IV mAb reactivity was assayed on trypsin digested GBM ELISA plates. Native fibronectin, trypsin digested SCM, pepsin digested collagen I and pepsin digested collagen IV were used as inhibitors of anti-collagen IV mAb reactivity with trypsin digested GBM. A level of 50% inhibition of anti-collagen IV mAb reactivity was chosen for comparison of extent of anti-collagen IV mAb reactivity and this data is summarized and presented in Table 5.

Percent Competitive Inhibition
of Anti-Collagen IV mAb Reactivity

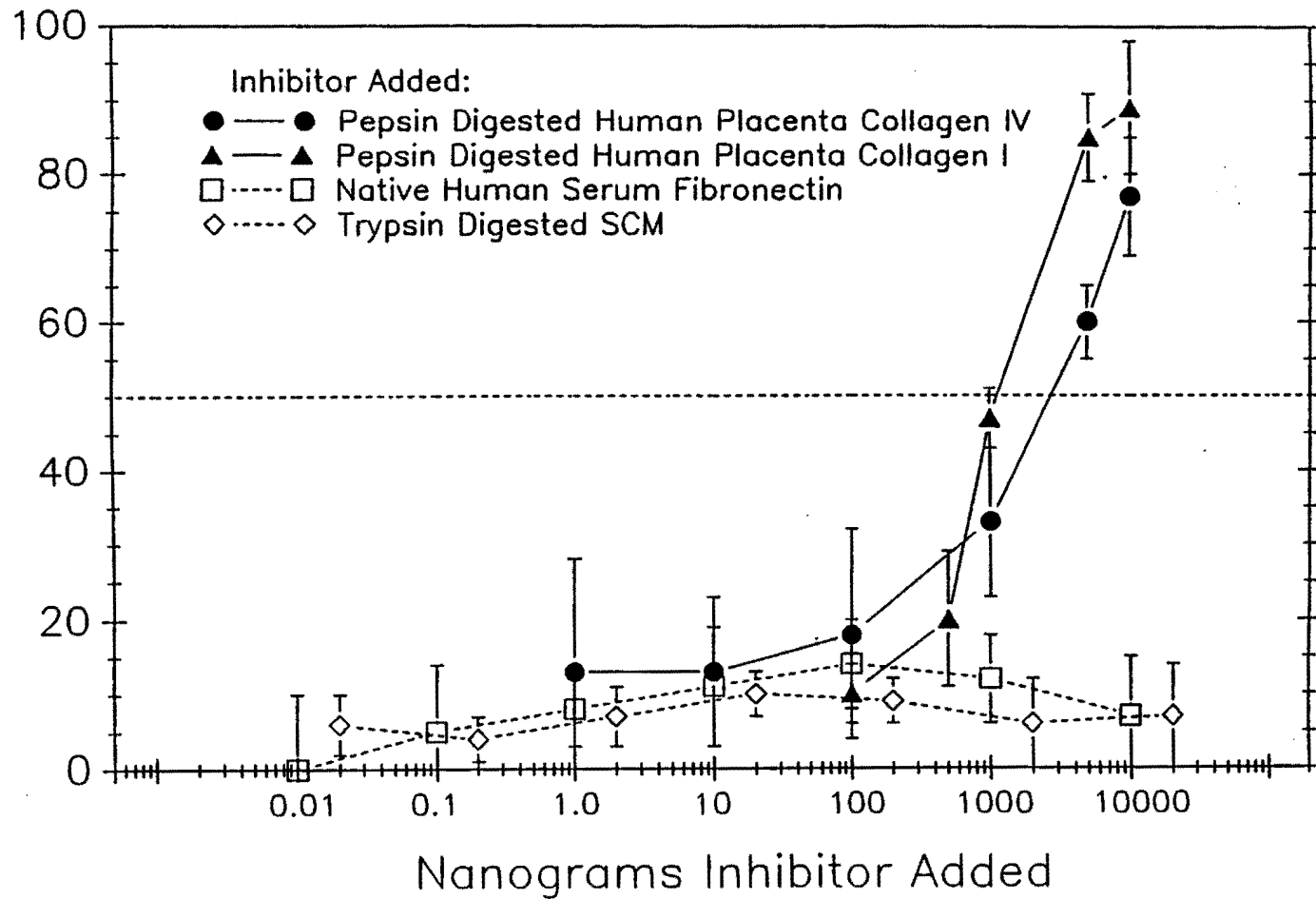


TABLE 5.

SUMMARY OF COMPETITIVE INHIBITION STUDIES
INHIBITION OF ANTI-COLLAGEN IV mAb REACTIVITY

Inhibitor	Nanogram amount of inhibitor required to achieve 50% inhibition of anti-collagen IV mAb reactivity (at 50 ng of anti-collagen IV mAb)
Trypsin digested SCM	NI
Human serum fibronectin	NI
Pepsin digested human placenta collagen I	1000 +/- 240 ng
Pepsin digested human placenta collagen IV	2050 +/- 530 ng

NI-- not inhibitory at 50% inhibition of anti-collagen IV mAb reactivity.

Nanogram inhibitory values were determined from a first order linear regression plot of each inhibitor and at 50% inhibition of anti-collagen IV mAb reactivity.

DISCUSSION

The long established clinical association between infection with a nephritogenic strain of streptococci and development of glomerulonephritis (Futcher, 1940; Stollerman, 1969) has led many researchers to speculate upon and examine possible etiologies for poststreptococcal glomerulonephritis. One hypothesis is that streptococcal component-antibody immune complexes deposit in the GBM or complexes form in situ (Friedman et al., 1984; Stinson et al., 1983). Another hypothesis is that antigenic relatedness exists between the streptococcal cell membrane and a GBM component (Markowitz and Lange, 1964; Lange, 1980b; Fitzsimons, Weber, and Lange, 1987; Zelman and Lange, 1989). A murine monoclonal antibody generated to type 12 SCM was used to investigate the relationship between SCM and GBM.

The first major hypothesis of this thesis is that an anti-SCM mAb (IIF4) can react with isolated, human GBM; the second hypothesis is that IIF4 will react with the majority of GBMs tested. IIF4 reacted with isolated, human GBM and IIF4 reacted with all 26 GBMs tested. In this study, the IIF4 reactive epitope in GBM was pepsin, collagenase, and trypsin insensitive. It was also noted that two

groups of GBMs were evident when extent of IIF4 reactivity with the panel of GBMs was examined.

The third hypothesis was that IIF4 would recognize a specific component of GBM. This mAb reacted with collagen IV. The fourth hypothesis was that IIF4 would recognize an identifiable location on collagen IV. Attempts were made to accomplish this and the data was consistent with the IIF4 reactive epitope was in fragments of collagen IV which had included part of the triple helical region and the NC1 globular domain. Comparisons were made between IIF4 and a conventional anti-collagen IV mAb; it was seen that these two antibodies did not recognize the same epitope; the anti-collagen IV mAb recognized epitope was collagenase sensitive. The hypotheses addressed in this dissertation were aimed at furthering our understanding of the incidence of this epitope in GBM.

While this evidence does not prove the role of SCM in the etiology of poststreptococcal glomerulonephritis, the demonstration of the reactivity of an anti-SCM mAb to a GBM antigen does support the hypothesis of antigenic relatedness between these two entities. These data will be discussed in relation to the two models of poststreptococcal glomerulonephritis development and the significance of reactivity of an anti-SCM mAb with collagen IV. A model will be presented for the generation of an anti-SCM mAb reactive with GBM and collagen IV and its possible roles in poststreptococcal glomerulonephritis.

Characterization and Reactivity of the anti-SCM mAb, IIF4

The first consideration in this study was the partial purification, quantitation and characterization of the anti-SCM mAb, IIF4. These qualitative and quantitative determinations are the basis for the use of IIF4 in all the studies in this dissertation.

IIF4 was isotyped as IgM (Table 1). Murine IgM is known to be a characteristically difficult monoclonal antibody with which to work. For example, murine IgM is not readily bound to Protein A or Protein G (Kronvall, Grey and Williams, 1970; Lindmark, Thoren-Tolling, and Sjoquist, 1983) which are commonly used to partially purify IgG antibody species. In this study, euglobulin precipitation was found to be an easy, quick and inexpensive means of partially purifying murine IgM. Albumin and transferrin are removed by euglobulin precipitation from the ascites and primarily IgM and IgG3 remain (Garcia-Gonzales et al., 1988). Purity of the euglobulin fraction was demonstrated by SDS-PAGE and calculated to be 65-85% on average.

An important consideration in working with murine IgM antibodies is that IgM are generally considered to be of low affinity. A hallmark of a primary immune reaction is a polyclonal IgM response. As the response matures to a secondary reaction, dogma states that a relatively few high affinity IgM clones are switched to IgG production and expanded while the low affinity clones are not expanded. Thus, the isolation of an IgM clone of low affinity is potentially high. Affinity data for IIF4 is unavailable. However, studies done on naturally occurring IgM

producing clones have found these mAbs to be multireactive (Rousseau, Mallett, and Smith-Gill, 1989). Naturally occurring IgM clones from murine neonates were also found to be multireactive for other IgM molecules (Holmberg et al., 1984). In the present study, IIF4 and TEPC 183 reactivity with a number of proteins is presented. TEPC 183 appears to bind to many proteins to some extent. The TEPC 183 ligand is not known, but this low level of binding to many proteins is probably due to the nature of the IgM molecule. The general rule for IgM affinity is low affinity, high valence number and high affinity, low valence number. It has been noted in some IgM molecules that approximately half the binding sites are relatively higher affinity than the other binding sites; the authors did not ascribe this to different combining sites in one molecule, but the effect of conformation of the decavalent IgM molecule (Pascual and Clem, 1988). Lower affinity sites could lead to increased nonspecific binding of the IgM antibody. However, TEPC 183 is used here as an isotypic match to control for any nonspecific binding of murine IgM which may be expected in these types of experiments. Statistical data is given to indicate when the IIF4 or TEPC 183 reactivity with a protein is significantly different.

IIF4 reactivity was characterized with its autologous antigen, SCM, and another streptococcal cell membrane from S. mutans. The use of the cell membrane from S. mutans was appropriate for the comparison with SCM since S. mutans cell membrane has been cited as having an immunological relationship with heart muscle (Van De Rijn, Bleiweis, and Zabriskie, 1976; Hughes et al., 1980) and

kidney tissue (Albini et al., 1985). Human anti-heart sera was capable of binding polypeptides from S. mutans cell membrane (Ayakawa et al., 1985). Disrupted S. mutans has been used in the generation of sera and the sera was capable of binding to human GBM and heart muscle and this reactivity could be absorbed by the cell membrane (Nisengard, Stinson, and Pelonero, 1983). S. mutans has also been implicated in the generation of glomerulonephritis. Disrupted streptococci injected into rabbits resulted in poststreptococcal-like kidney pathology and deposition of S. mutans antigens in the GBM (Albini et al., 1985). Thus, evidence does exist that S. mutans, possibly the cell membrane, and heart and kidney tissue share a common antigen.

IIF4 was found to react significantly with SCM and not with S. mutans cell membrane (Figure 1). Furthermore, TEPC 183, was found to react significantly less with SCM than IIF4 did with SCM. Competitive inhibition of IIF4 reactivity to SCM was tested with SCM and S. mutans cell membranes as inhibitors (Figure 2). SCM did inhibit IIF4 reactivity with SCM while S. mutans cell membrane was unable to inhibit IIF4 reactivity to any extent distinguishable from background. These two sets of data confirmed that the IIF4 reactive epitope resides in its homologous antigen, SCM, and not in another streptococcal cell membrane from S. mutans.

Characterization of GBMs and IIF4 Reactivity with GBMs

The first major hypothesis of this thesis was that IIF4, an

anti-SCM mAB, reacts with GBM. In indirect immunofluorescence, IIF4 was found to bind to renal cortical GBM, mesangial matrix and tubular basement membrane (Figure 3A). The green immunofluorescence due to IIF4 that stained the GBM, mesangial matrix, and tubular basement membrane was of a spotty or hazy, granular nature. Others have reported a granular type pattern seen in human kidneys using a monoclonal antibody generated to cultured glomerular cells (Nakamura, 1986). Antibodies derived from patients with acute poststreptococcal glomerulonephritis stained glomeruli from these patients in a granular pattern (Yoshizawa *et al.*, 1973). The localization of fluorescence in the renal cortical tissue is consistent with the IIF4 recognized epitope as a component of the GBM, tubular basement membrane, and in association with the mesangial cells.

Autofluorescent (yellow-orange) mesangial nuclei were found in all renal tissues examined regardless of antibody type used in the experiment. The autofluorescence is helpful in recognizing anatomical structures; for example, the unique cellular array of the glomeruli or tubules is readily evident. This condition may be due to the variable time interval between demise, autopsy and freezing of the kidney tissue. Autodegradation is commonly known to occur and the variable intensity of nuclear autofluorescence may be due to breakdown and exposure of nuclear compounds in the tissues.

As a comparison, anti-collagen IV mAb was used to stain human kidneys; a linear pattern was seen. Other anti-GBM sera have

stained GBM in linear patterns. Acid eluates from kidney biopsies of patients with Goodpasture's syndrome yielded antibodies reactive with GBM in a linear fashion (Lerner, Glasscock, and Dixon, 1967). Monoclonal antibodies developed to collagenase solubilized GBM also produced linear patterns along the GBM (Pusey et al., 1987). When a heterologous rabbit anti-human GBM anti-serum was generated, it stained human kidney tissue in a linear pattern with intense staining of the inner endothelial zone and outer epithelial zone with mesangial matrix involvement (Fish et al., 1983). Antibodies from a patient with anti-GBM nephritis, as well as an anti-NC1 sera, produced a linear staining pattern on normal human kidney sections (Thorner et al., 1989). Antiserum to components of the GBM such as heparan sulfate proteoglycan produced intense linear patterns along the GBM (Van Den Heuvel et al., 1989). Clinically, the type of fluorescence seen is used as a diagnostic sign and are classed as starry sky (fine granular deposits of IgG/IgM and C3), mesangial (C3 deposits), or garland (IgG and C3 deposits) (Sorger et al., 1983; Rodriguez-Iturbe, 1984). The ultrastructural significance of any type of staining is unknown, but each type may reflect a distinct antibody reactivity localization with a component or components of the GBM.

In order to characterize the indicated IIF4 reactivity in the GBM, GBM was isolated from 26 human kidneys (Greenspon and Krakawer, 1950; Carlson et al., 1978). A common method of GBM characterization is amino acid analysis (Wieslander, 1983; Spiro, 1967; Kefalides, 1972; Lange, 1969). While this method cannot determine the identity

of proteins comprising the GBM, this method can demonstrate characteristic amino acids of known proteins. The 25 GBMs assayed for amino acid composition revealed the presence of hydroxyproline and hydroxylysine (Table 2); these amino acids are found only in collagenous proteins. The amino acid composition analysis of the 25 GBMs is consistent with previously reported GBM analyses in that hydroxyproline, hydroxylysine and a high glycine level (greater than or equal to 10%) are present (Wieslander, 1983; Westberg and Michael, 1970).

The panel of GBMs was further characterized by reactivity with IIF4. All 26 individual GBM preparations reacted with IIF4. These data indicate that all GBMs (in this study) contain the IIF4 recognized epitope. This study was differentiated from other studies of antibody reacting with GBM by using a panel of 26 human GBMs and examined anti-SCM mAb reactivity to each GBM. Previously, studies have been conducted with human GBM using a pool of several GBMs (Westberg and Michael, 1970; Wieslander, 1983; Lange, 1980b; Markowitz and Lange, 1964). The reactivity of anti-SCM sera with individual GBMs was also studied (Blue and Lange, 1976; Lange, 1969; Lange, 1980a). These studies indicated that an anti-SCM sera could react with glomeruli in tissue sections, but since sera were used, the shared epitope concept could not be invoked. This study was also unique in that the panel of human GBMs was screened for reactivity with an anti-SCM mAb. Other studies have looked at the reactivity of GBM components with a patient's serum; this is especially true for reactivity of Goodpasture's patients serum

reacting with the NC1 domain of collagen IV which was derived from a pool of human GBM tissue (Pusey et al., 1987; Weber, Meyer zum Buschenfelde and K hler, 1988; Fish et al., 1984). Extensive studies have characterized Goodpasture's patient serum reacting specifically with monomers of 26 kDa and dimers of 52 kDa derived from the NC1 domain of collagen IV. This antigen is the best characterized glomerular polypeptide involved in autoreactivity.

Other studies have characterized the GBM with monoclonal antibodies reactive with protein components of the GBM such as laminin (Kefalides et al., 1986) and heparan sulfate proteoglycan (Van Den Heuvel et al., 1989; Edge and Spiro, 1987). These studies have sought to define the structure and the role of the individual components in a basement membrane complex. In distinction, studies with anti-SCM mAb seek to characterize the reactivity of such antibodies with human GBMs and focus on the presence of the IIF4 reactive epitope in that GBM component.

Relatively high and low IIF4-reactive groups of GBMs could be seen. There are several possible explanations for this variability seen in extent of IIF4 reactivity (Figures 7, 8, and 9). The GBMs used in this study were each isolated from human kidneys. Each kidney was collected and frozen upon autopsy at variable times after death. This is an uncontrolled factor in these experiments; it is well known that autolysis will take place in tissue after demise and evidence of autodegradation was seen in the autofluorescence of mesangial nuclei in all tissues examined. However, each GBM was isolated and enzyme digested under equivalent conditions. Variable

amounts of autolysis had most likely taken place in each kidney prior to GBM isolation and enzyme digestion. The IIF4 reactive epitope may be more readily available for binding in some GBM preparations than in others. This may be due to the complex structure of the components of GBM when the GBM is enzyme solubilized. The IIF4 reactive epitope may occur in varying densities in each GBM. These experiments can not distinguish among these possibilities of the variation seen of IIF4 reactivity with the GBMs.

IIF4 also reacted equivalently with pepsin, collagenase or trypsin digested GBM (Figure 7, panel B); thus, the IIF4 reactive epitope was trypsin, collagenase, and pepsin insensitive. Bacterial collagenase specifically cleaves the peptide bond in collagen molecules between the "Y" and the glycine residues in the "Gly-X-Y-Gly-X-Y" repeating triplet of the triple helical of the collagen molecule (Harper, 1980). Trypsin is highly specific for positively charged side chains with arginine and lysine (Brown and Wold, 1973). Pepsin cleaves proteins at aromatic amino acids (Ryle, 1970). The data generated here indicated that the IIF4 reactive epitope was not part of the triple helical region of collagen, did not have an arginine/lysine within or near the epitope, and did not contain aromatic amino acids.

Characterization of IIF4 Reactivity with Components of GBM

The next goal of this dissertation was to identify the IIF4 reactive GBM component. This was accomplished by examining major

components of the GBM for IIF4 reactivity. IIF4 reacted to an equivalent extent with collagenase digested collagen IV and collagen I, with trypsin digested collagen I, and with pepsin digested collagen I and collagen IV (Figure 7). This data clearly targets the IIF4 reactive protein as collagen. In order to confirm IIF4 reactivity, another method was employed.

IIF4 reactivity with enzyme digested GBMs, enzyme digested collagen I and collagen IV, and other major components of GBM was evaluated by competitive inhibition. Trypsin, collagenase or pepsin digested GBM all inhibited IIF4 reactivity significantly. Twice the amount of collagenase or pepsin digested GBM was required to achieve the same extent of inhibition as trypsin digested GBM. Possible explanations of this result are that more IIF4 reactive epitopes exist in the trypsin digested GBM or trypsin digestion best opens up the quaternary structure of the GBM to reveal IIF4 reactive epitopes. In any case, all enzyme digests of GBM significantly (at least 40%) inhibited IIF4 reactivity with SCM.

Low molecular weight, trypsin digestion derived, murine GBM polypeptides were found to competitively inhibit anti-SCM mAb reactivity with SCM peptides (Zelman and Lange, 1989). It was seen that 20 pM of a 5 kDa GBM peptide completely inhibited the reactivity of 12.5 pM of anti-SCM mAb. The interpretation of these and other data was that the 5 kDa fragment contained two epitopes available for anti-SCM mAb binding. These studies also stressed the importance of the size of the epitope containing fragment. As the epitope containing fragment increased in size, the more steric

hindrance of binding by the paratope became important. In the present study, the native size of the pepsin or collagen digested collagen IV polypeptide bound to IIF4 is unknown, but if the polypeptides are assumed to be an average of 200 kDa, 5.8 pM of pepsin digested collagen IV were required to inhibit (at 40%) 1 pM of IIF4. Likewise, 20 pM of collagenase digested collagen IV were required to inhibit (at 40%) 1 pM of IIF4. The polypeptides from the pepsin digests are probably large, since extensive reduction is required to produce smaller (~100 kDa) pepsin fragments (Glanville et al., 1985). Interference of paratope binding due to steric hindrance of large (~200 kDa) polypeptides may explain the large amount of antigen needed to inhibit antibody activity. These results confirmed that IIF4 exhibited specific binding to human GBM and to a collagen molecule.

Other major components of the GBM were used as competitors of IIF4 reactivity with SCM. Neither human serum fibronectin, bovine kidney heparan sulfate proteoglycan nor murine sarcoma derived laminin could inhibit IIF4 reactivity with SCM to any extent distinguishable from no inhibition of IIF4 reactivity. Fibronectin was used in these studies since fibronectin can be associated with collagen IV, although not a native part of GBM (Mauger et al., 1987; Hahn et al., 1980). These results demonstrated that these major components of the GBM did not contain the IIF4 reactive epitope.

IIF4 had reactivity with collagenase, trypsin, or pepsin digested collagen I (Figure 7) which is not a reported constituent of GBM (Timpl, 1989). Pepsin digested collagen I was unable to

competitively inhibit IIF4 reactivity (Figure 8). Solution based competitive inhibition is a common method used to confirm antibody reactivity (Zelman and Lange, 1989; Dale and Beachey, 1985; Cunningham et al., 1989; Kraus et al., 1989). Solid phase assays are considered more sensitive for high affinity antibodies while fluid phase assays, such as a Farr assay, are considered more sensitive for lower affinity antibodies (Kennel, 1982; Nimmo et al., 1984; Butler et al., 1978; Peterman, Voss and Butler, 1985). In these assays, a fluid phase assay, competitive inhibition, may be more reliable for determination of murine IgM reactivity. Epitopes shared by collagen types have been easily detectable by ELISA and Western blot. In a study of familial nephritis, IgA patient serum reacted with the triple helical domain of collagens I, II, and IV, indicating that antibodies directed to this moderately conserved domain will have a wide span of reactivity with types of collagen (Cederholm et al., 1986). Thus, based on the isotype used and the reported sensitivity of these assays when using IgM, it seems unlikely that collagen I is a IIF4 target antigen.

IIF4 reactivity with SCM was significantly inhibited by enzyme digests of collagen IV. Collagenase digested GBM or collagen IV inhibited IIF4 reactivity with SCM to approximately the same extent. This may be due to the fact that GBM and isolated collagen IV are somewhat resistant to collagenase digestion (Cederholm et al., 1986; Risteli et al., 1980; Siebold et al., 1987). The products of collagenase digestion of collagen IV are large (~100 kDa) polypeptide chains which are highly associated with one

another. Possibly, collagenase digestion of GBM or collagen IV reveals a limited number of IIF4 reactive epitopes even though collagen IV was purified away from other placental basement membrane constituents.

Three times the amount of pepsin digested GBM as pepsin digested collagen IV was needed to inhibit IIF4 reactivity to the same extent. Pepsin digests of isolated collagen IV are commonly used to study collagen IV polypeptides (Glanville et al., 1985; Qian and Glanville, 1984; Glanville, Rauter, and Fietzek, 1979) and it is known that pepsin digestion of collagen IV produces large (~95 kDa) polypeptide chains (Qian and Glanville, 1984; Glanville et al., 1985). This data suggested an approximate 3 fold increase or enrichment of available IIF4 reactive epitopes in isolated, pepsin digested collagen IV as compared to pepsin digested GBM. Pepsin digested collagen IV inhibited IIF4 reactivity with SCM the greatest extent among all enzyme digests of GBM and collagen IV assayed. Trypsin digested SCM and pepsin digested collagen IV were able to inhibit IIF4 reactivity to pepsin digested collagen IV (Figure 9). Pepsin digested collagen IV could achieve 40% inhibition of IIF4 reactivity with amounts of pepsin digested collagen IV comparable to those needed to inhibit IIF4 reactivity with SCM (Table 3). However, twice as much SCM was required to inhibit IIF4 reactivity with pepsin digested collagen IV as with SCM. This may be due to better availability of the IIF4 reactive epitope in the complex of pepsin digested collagen IV polypeptides as compared to the complex of trypsin digested SCM polypeptides. This trend was also apparent

in the collagen IV inhibition of IIF4 reactivity with SCM (Table 3). These experiments demonstrated that collagen IV contained the IIF4 reactive epitope and these results indicated that the binding availability or incidence of available epitopes was greater in pepsin digested collagen IV polypeptides than in trypsin digested SCM polypeptides.

Characterization of IIF4 Reactive Polypeptides in GBM and Collagen IV

The next purpose of this dissertation was to identify the portion or domain of collagen IV reactive with IIF4. The method chosen to answer this question was immunoprecipitation of enzyme digested GBM, collagen IV, and other GBM components by IIF4 and goat-anti-mouse IgM coupled agarose and separation on SDS-PAGE. This was the most economical, technically simple and widely accepted method available for demonstrating reactions of an antibody with specific proteins, polypeptides, or peptides (Weir, 1986; Chen et al., 1986). In this type of experiment, the eluted, immunoprecipitated material contains IgM, IgG derived from the goat anti-mouse agarose beads, and proteins bound to IgM, if any. Thus, the eluted material contains mu chain (65 kDa), gamma chain (51 kDa), light chains (30 kDa), J chain from IgM (16 kDa), and any immunoprecipitated proteins. Results presented contain a representation of material eluted from the goat anti-mouse IgM agarose beads/antibody complex as well as the beads alone and demonstrated on silver stained SDS-PAGE. Material eluted from the

goat anti-mouse IgM agarose beads contains polypeptides other than only gamma and light chain; polypeptides of 37, 40, 44, 55, and 66 kDa have been detected (Figures 10, 11B, 12, and 14). These polypeptides are of unknown origin, but are derived from the beads or antibodies themselves. Polypeptides of these molecular weights are not considered immunoprecipitated when they are present in the representation of a IIF4 or TEPC 183 immunoprecipitated sample. Polypeptides which appear in IIF4 immunoprecipitated sample as well as in the TEPC 183 immunoprecipitated control are considered nonspecific. Immunoprecipitated proteins of molecular weight equal or similar to any of the antibody chains may be undetected. This method can, however, detect immunoprecipitated polypeptides that are reproducibly present and of a molecular weight distinct from the antibody chains.

As summarized in Table 4, the IIF4 immunoprecipitation of polypeptides from collagenase or pepsin digested GBM, collagen I, collagen IV and laminin indicated that IIF4 generally reacted with GBM and collagen IV polypeptides of 100-200 kDa. In four GBM samples, other IIF4 reactive polypeptides from collagenase digested GBM of lower molecular weight were seen (Figure 10, 11 B, and 14). IIF4 reacted with ~200, 140, 100, and 60 kDa polypeptides from pepsin digested GBM (Figure 15). It was also suggested that IIF4 was reactive with polypeptides of ~200, 140, and 90 kDa from pepsin digested collagen IV (Figure 17A); in this experiment, TEPC 183 was seen to have some reactivity with these collagen IV polypeptides. IIF4 did not react with polypeptides from pepsin digested collagen I

or native laminin (Figure 17B). With the exception of pepsin digested collagen IV polypeptides, these results strongly supported and were in agreement with data that demonstrated the IIF4 reactive GBM component was collagen IV.

Although these experiments indicated IIF4 reactive polypeptides of similar or equivalent molecular weight from digests of collagen IV or GBM, these experiments did not identify the domain of collagen IV reactive with IIF4. Collagenase digests of GBM and of collagen IV contain polypeptides of 54 and 26 kDa that are reactive with anti-Goodpasture's antibody (Pusey et al., 1987; Weber et al., 1987). An antigen reactive to Goodpasture antibody has been identified as residing in a 26 kDa monomer of the NC1 globular domain of collagen IV (Pusey et al., 1987; Butkowski et al., 1985; Wieslander, Kataja, and Hudson, 1987). IIF4 reacted in only one case with a 27 kDa polypeptide from collagenase digested GBM (Figure 14), but this was not seen in IIF4 reactivity with collagenase digested collagen IV. A 45 kDa polypeptide from collagenase digested collagen IV was IIF4 reactive, but this molecular weight has not been reported for the dimer form of the Goodpasture's antigen (Pusey et al., 1987). This indicates that the IIF4 reactive epitope is probably not the Goodpasture's antigen; however, this does not rule out other possible epitopes in the NC1 globular domain of collagen IV as IIF4 reactive.

Interstitial collagen I and basement membrane collagen IV differ in tissue distribution (Kuhn, 1987), but share sequence similarity in their triple helical regions. This large domain in

both types of collagen is predominantly "Gly-X-Y" repeating triplets in three chains that are wound around each other. There is a small amount of dissimilarity amongst the triple helical domains of collagen I and collagen IV. The most striking difference between the two collagen types is that collagen I has no globular domains at its amino and carboxyl termini. IIF4 was not reactive with collagen I in competitive inhibition with IIF4 or immunoprecipitation with IIF4. This suggested that IIF4 was reactive with a globular domain in collagen IV. The 7S and NCl globular domains are the largest globular domains in collagen IV and are structurally prominent (Yurchenco, 1990; Yurchenco et al., 1986; Yurchenco and Furthmayr, 1984). Human collagen IV alpha 1(IV) chain does contain 21 globular-like interruptions of its triple helical domain that are 8 amino acid residues or larger; the human alpha 2(IV) chain has 23 similar globular interruptions of its triple helix. This property is unique to collagen IV (Brazel et al., 1988). These globular domains are possible candidates for the location of IIF4 reactivity as suggested by these studies.

IIF4 reactive polypeptides from pepsin digested GBM were 100 and 85 kDa. Pepsin digestion and reduction of collagen IV yields polypeptides of 140 (alpha 1 chain), 120 (alpha 2), 100 (alpha 1), 75 (alpha 2), 50, and 25 kDa (Qian and Glanville, 1984; Brazel et al., 1988; Glanville and Rauter, 1981). These polypeptides from pepsin digested collagen IV have been identified as entire or a slightly truncated versions of the two types of alpha chains commonly found in collagen IV (Qian and Glanville, 1984; Glanville

et al., 1985). The 100 kDa polypeptide from pepsin digestion of collagen IV has been sequenced (Qian and Glanville, 1984). Although there has been much confusion in the literature, it has been determined that these polypeptides originate in the NC1 globular domain (Glanville, 1987). IIF4 was not shown to specifically immunoprecipitate polypeptides of these molecular weights from pepsin digested collagen IV; TEPC 183 had some reactivity with these polypeptides (Figure 17A). However, the determination of collagen IV as the GBM protein reactive with IIF4 and the demonstration of pepsin digested GBM polypeptides of 100 and 85 kDa as IIF4 reactive targeted similar collagen IV polypeptides as containing the IIF4 reactive epitope.

In studies using an mAb to type IV collagen, reduced polypeptides from a crude pepsin digestion of collagen IV were found to be 180, 140, 120, 90, 40, and 15 kDa with a nonreduced size of 260 kDa (Kino et al., 1988). This mAb did not react with collagen I, collagen V, collagen III, collagenase digested collagen IV, fibronectin or laminin. It did react with denatured collagen IV indicating that the epitope was not part of the triple helical conformation or conformation defined. Amino acid analysis of the native pepsin digested collagen IV polypeptide reactive with this mAb indicated 15 cysteine residues in this polypeptide. The authors speculated that these pepsin digest fragments originated in the NC1 domain.

In another study, a mAb generated to collagen IV was found to react with a high molecular weight (250 kDa) polypeptide that

could be localized to placenta, bovine lens capsule, and GBM. This polypeptide was pepsin and collagenase resistant and distinct from alpha 1(IV) and alpha 2(IV). Anti-serum to this polypeptides stained the GBM in a linear pattern with mesangial involvement. The authors postulated that this may be a new alpha chain or possibly the alpha 4(IV) (Scheinman and Tsai, 1984). Both of these studies indicate that collagen IV is a highly complex molecule with many potential sites that are capable of eliciting antibody generation.

Sequencing of the IIF4 reactive polypeptides to identify the IIF4 epitope was impractical. The polypeptides were too large for sequencing and too large to allow for the identification of the IIF4 reactive epitope. However, in order to support the hypothesis that the IIF4 reactive polypeptides originated in the NC1 domain, the IIF4 reactive polypeptides were isolated and subjected to amino acid analysis. The IIF4 reactive polypeptides from collagenase digested GBM or collagen IV (120 and 100 kDa) were similar in the content of some amino acids. Of particular note is the cysteine residue content. In a 120 or 100 kDa polypeptide, a 2-4% cysteine residue content corresponds to approximately 13-32 cysteine residues. Similarly, the IIF4 reactive polypeptide from pepsin digested GBM and the equivalently sized polypeptide from pepsin digested collagen IV had high cysteine content (30 residues). High cysteine content was previously noted in peptide mapped GBM fragments (Lange, 1969). This data is consistent with the IIF4 reactive polypeptides in GBM, and potentially in collagen IV, as consisting of part of the triple helical and NC1 domains.

Reactivity of Anti-Collagen IV mAb with GBM

Having shown that IIF4 was reactive with collagen IV, it was prudent to compare its reactivity to that of an anti-collagen IV mAb and examine differences and similarities in their reactivity with GBM and GBM components. Significantly, anti-collagen IV mAb reacted with GBM differently than did IIF4 in indirect immunofluorescence. A linear pattern of antibody binding along the GBM was seen with anti-collagen IV mAb while IIF4 stained the GBM in a granular pattern. Additionally, other whole anti-GBM anti-sera are known to stain GBM in a linear pattern (Thorner et al., 1989; Fish et al., 1984). These data suggest that the IIF4 recognized epitope in GBM was different than the epitope recognized by anti-collagen IV mAb.

Anti-collagen IV mAb did not react with trypsin digested SCM, S. mutans cell membrane, laminin or fibronectin; this was the expected result. Anti-collagen IV mAb did react with isolated, trypsin or pepsin digested GBM or collagen IV, but not with collagenase digested GBM or collagen IV. These results indicate that the anti-collagen IV mAb recognized epitope was collagenase sensitive. The IIF4 recognized epitope, however, was not collagenase sensitive. Collagenase digestion of collagen IV and GBM is known to be incomplete (Risteli et al., 1980). Possibly the anti-collagen IV mAb reactive epitope is part of the interwinding superstructure of the three alpha chains of collagen IV at exposed sites and can be cleaved by collagenase treatment. Again, these

data suggested that the IIF4 reactive epitope was not part of the Gly-X-Y repeating unit.

Anti-collagen IV mAb also reacted with pepsin digested collagen I. At 50% inhibition of anti-collagen IV mAb reactivity, twice the amount of collagen IV was required to inhibit the mAb as was required of collagen I. This may be due to the availability of the epitope in these two collagens.

These data, along with the inability of anti-collagen IV mAb to react with collagenase digested GBM or collagen IV indicated that the anti-collagen IV mAb epitope was located on the triple helical region of collagen molecules. In contrast, it was suggested that IIF4 reactivity could not be inhibited by collagen I, but did react with collagenase digested GBM or collagen IV. These two mAbs clearly recognized different epitopes in the same collagen molecule.

Significance of IIF4 Reactivity with Collagen IV

The reactivity of IIF4 with collagen IV is significant in two areas; molecular mimicry and development of glomerulonephritis.

1. Significance of anti-SCM mAb reactivity with collagen IV in molecular mimicry.

The concept of molecular mimicry is based on the antigenic relatedness of two distinctly different antigens. Many autoimmune diseases have been implicated as having a basis in molecular mimicry. The classic example of this is of ankylosing spondylitis (AS). The development of arthritis in AS has been linked to

infection with *Salmonella*, *Shigella*, *Yersinia*, and especially with *Klebsiella pneumoniae*. The HLA B27 molecule and the *K. pneumoniae* nitrogenase were reported to share an epitope (Schwimmbeck, Yu and Oldstone, 1987); this shared epitope would explain the high incidence of AS patients who are HLA B27⁺. Other bacterial proteins have been implicated in sharing epitopes with mammalian tissue and correlated with incidence of autoimmune disease. Evidence supports a shared epitope between streptococcal M proteins and cardiac myosin (Krisher and Cunningham, 1985; Dale and Beachey, 1985) and this relationship has been implicated in the development of rheumatic fever. Hepatitis B virus polymerase and the encephalitogenic site of myelin basic protein are believed to share an epitope and be involved in the etiology of experimental allergic encephalomyelitis (EAE) (Fujinami and Oldstone, 1985). Ankylosing spondylitis, rheumatic fever, and EAE are all be thought to be caused by foreign antigens which share epitopes with host tissue; infection with these agents may induce an autoimmune response. This study indicated that anti-SCM mAb, IIF4, reacted with collagen IV; this adds more evidence for the existence of molecular mimicry which could theoretically lead to an autoimmune response.

2. Development of poststreptococcal glomerulonephritis, (PSGN)

One theory of the etiology of PSGN is that immune complexes are formed in the circulation or in situ at the GBM with streptococcal antigens (Cameron, 1982; Rodriguez-Iturbe, 1984; Friedman et al., 1984). In this model, circulating streptococcal

antigens bind with IgG or IgM and are deposited in the kidney. Complement is activated; vasoactive peptides are released and mediate vascular permeability, chemotaxis and activation of neutrophils. Neutrophils enter the tissue and attempt to phagocytize the immune complexes. If phagocytosis is not accomplished due to size, location or tightness of binding of the immune complex to the GBM, the neutrophil releases its lysosomal enzymes and GBM damage results (Williams, 1987). This leads to loss of GBM integrity and renal function failure.

The data presented here does not support or refute the immune complex model for development of poststreptococcal glomerulonephritis, however, it does support a second model. In the second model, antibodies evoked by streptococcal antigens are directed to a common epitope located in the GBM (Lange, 1980a; Markowitz and Lange, 1964). A monoclonal antibody, IIF4, generated to type 12 SCM was shown to react with collagen IV. IIF4 reactivity with collagen IV alone does not prove, but indicates that SCM and GBM share an epitope. Formal proof of a shared epitope requires the isolation of and sequencing of small, IIF4 reactive peptides from GBM and SCM, but attempts to date have been unsuccessful (Zelman and Lange, 1989).

Collagen IV is found in all basement membranes: skin, placenta, kidney, gut, lung, cornea and blood vessels (Glanville, 1987). Thus, the reactivity of anti-SCM mAb with collagen IV has implications beyond that of reactivity with glomerular components alone; other basement membranes may be targets for anti-SCM

antibodies present after streptococcal infection or autoantibodies. It is possible that a IIF4-like antibody could bind to the epitope in collagen IV in another basement membrane. Upon basement membrane damage, an immune complex of IIF4-like antibody and collagen IV fragment could be carried to and lodged in the GBM. This is another mechanism by which GBM may be damaged by a IIF4-like antibody, but this model, in an immune complex with a self antigen.

In this study, IIF4 reacted with all the panel of isolated GBM from normal kidney tissue. Presumably, not all 26 tissue sources were infected with type 12 streptococci at the time of demise; no overt kidney pathology was noted. It is possible that the epitope recognized is present in all GBMs. This study indicated 100% incidence of the epitope in the GBMs tested. A precedent for the widespread occurrence of an basement membrane autoantigen is the Goodpasture antigen. It too appears ubiquitously in basement membrane containing tissues and is present in bovine basement membranes (Hudson et al., 1989). The Goodpasture antigen appears to be required for renal integrity and function; a strain of Samoyed dogs who have been shown to lack the Goodpasture antigen frequently present with renal failure and complete loss of integrity of the GBM (Thorner et al., 1988). There is no strong indication from this present study that the IIF4 reactive collagen IV epitope is or is not the Goodpasture antigen. However, injection of a related anti-SCM mAb or intraperitoneal injection of the antibody-producing hybridoma cells into uninfected mice caused lung and kidney pathology highly similar to that found in patients with Goodpasture

syndrome (Fitzsimons and Lange, 1991). This may indicate that the IIF4 reactive epitope is related to the Goodpasture antigen and this awaits further study.

These data support the model of a common epitope between SCM and the GBM component, collagen IV, and the autoimmune component of poststreptococcal glomerulonephritis.

Model for development of poststreptococcal glomerulonephritis: GBM damage caused by antibodies reactive with a common epitope in SCM and GBM

Host protection from infection with a nephritogenic strain of streptococci involves bacterial elimination by opsonization. Host failure to produce opsonizing antibodies or subsequent release of streptococcal components after phagocytic bacterial destruction would allow the host to produce antibodies against streptococcal cell membrane antigens. These antibodies may arise from newly stimulated pre-existing autoreactive clones or they may arise de novo. This model predicts that IIF4 reactive epitope need not be sequestered, as is the belief with the Goodpasture antigen, since a foreign antigen evokes the antibodies. This may imply that a form of control over the development of autoreactive antibodies to the IIF4 reactive epitope is operating (such as suppression of autoreactive clones). GBM damage, loss of integrity and renal failure would follow the same course as described for the immune complex model. However, the anti-SCM antibodies are specific for an self antigen and thus are autoantibodies. Anti-SCM antibodies may

bind directly to collagen IV in the GBM or collagen IV in other basement membranes in the body and may lodge in the GBM as an immune complex with a fragment of collagen IV as an autoantigen. Anti-collagen IV reagents are directed to the majority of the GBM in immunofluorescence. IIF4, however, bound to GBM in a granular and less contiguous pattern. This may indicate potential initiation sites of GBM damage if autoantibodies are evoked. The GBM is exposed to the vascular flow by the fenestration of the endothelia; it is not privileged and is available for binding autoantibodies. Collagen IV comprises the majority of the GBM and it is an integral part of the structure and function of the GBM. Damage to the GBM and subsequent loss of renal function is a serious, possible consequence of host response to an epitope shared by SCM and GBM.

SUMMARY

The etiology of poststreptococcal glomerulonephritis is unknown, but development of glomerulonephritis after infection with a nephritogenic strain of streptococci has long been clinically correlated. The major purpose of this thesis was to investigate the antigenic relatedness between group A type 12 SCM and human GBM.

Anti-type 12 SCM mAb, IIF4, was specifically reactive with its autologous antigen, SCM, and not cell membrane from S. mutans. SCM was hypothesized to contain an epitope also expressed in GBM. This hypothesis was substantiated by experiments demonstrating IIF4 reactivity with GBM, mesangial matrix and tubular basement membrane in human renal cortical tissue in indirect immunofluorescence. IIF4 reacted with all individuals in a panel of 26 isolated, enzyme digested human GBMs to a significantly greater extent than an isotype matched control did with all GBMs. IIF4 reacted to an equivalent extent with trypsin, collagenase or pepsin digested GBMs indicating that the IIF4 recognized epitope is insensitive to these enzymes. Two populations of IIF4 highly and moderately reactive GBMs were noted.

IIF4 was hypothesized to react with a component of GBM. IIF4 was established to react with collagen IV, a major component of GBM, and not with laminin, fibronectin, heparan sulfate proteoglycan, or a related collagen, collagen I. This data also suggested that the IIF4 recognized epitope may reside in a globular portion of collagen IV. An attempt was made to isolate the IIF4 reactive component of collagen IV and of GBM. Size and amino acid content, especially cysteine content, of pepsin digested fragments of GBM were consistent with the polypeptides consisting of a portion of the triple helical domain and of the NC1 domain of collagen IV.

IIF4 reactivity and anti-collagen IV mAb reactivity with isolated GBMs and collagen IV was compared. The anti-collagen IV mAb recognized epitope in GBM, collagen IV, and collagen I was collagenase sensitive; this suggested that the anti-collagen IV mAb recognized epitope is located in the collagen triple helix. IIF4 and anti-collagen IV mAb recognized distinctly different epitopes.

The significance of an anti-SCM mAb reacting with human collagen IV and human GBM is discussed as it pertains to molecular mimicry. The importance and significance of a common epitope between SCM and GBM is discussed in relation to development of poststreptococcal glomerulonephritis. A model of poststreptococcal glomerulonephritis is discussed with collagen IV as the autoimmune target reactive with anti-SCM mAb.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

5 March 1991
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