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Isolation and Immunochemical Characterization of Streptococcal Cell Membrane Antigens Immunologically Related to Basement Membrane

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ISOLATION AND IMMUNOCHEMICAL CHARACTERIZATION OF STREPTOCOCCAL CELL MEMBRANE ANTIGENS IMMUNOLOGICALLY RELATED TO BASEMENT MEMBRANE

by

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The author dedicates this dissertation to the loving memory of his father, John.

VITA

The author, Mark Edward Zelman, is the son of John Zelman and Genevieve (Wozniak) Zelman. He was born August 23, 1963, in Chicago, Illinois.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
VITA	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
INTRODUCTION	1
LITERATURE REVIEW	3
Post-streptococcal Disease	3
Models for the Etiology of APSGN	5
Characterization of Streptococcal Cell Membrane	17
Specific Aims of Present Work	21
MATERIALS AND METHODS	23
Monoclonal Antibody	23
Basement Membrane Antigens	27
Streptococcal Antigens	28
C1q Purification	31
Protein Determinations	32
High Pressure Liquid Chromatography	33
HPLC-affinity Chromatography	34
Electrophoresis	36
Immunoblot and Western Blot of Soluble SCM Antigens	37
Enzyme-linked Immunosorbant Assay	39

Competitive Inhibition 39

Comparison of Native and Denatured Polypeptide Antigens .. 40

Analysis of mAb/antigen Interactions by Equilibrium Dialysis 40

Amino Acid Analysis 44

RESULTS 46

Monoclonal antibody isolation and purification 47

Basement membrane isolation and purification 47

Streptococcal cell membrane isolation and purification ... 52

Isolation of Membrane Antigens by Affinity Chromatography 52

Immunologic Analysis of SCM Polypeptide Antigens 63

Affinity of mAb for SCM polypeptide Antigens 77

Comparison of Native and Denatured SCM Antigens 84

Amino Acid Analysis 86

Amino Acid Sequencing 86

DISCUSSION 91

Immunologically related antigens are present in SCM and BM 91

SCM antigens exhibit size-related reactivity 94

Monoclonal antibody and valence are related to antigen size 98

Reactivity of SCM antigens following denaturing conditions 101

Amino acid composition is related to antigen binding characteristics 103

A proposed structure for the SCM antigen 104

A proposed role for these antigens in APSGN 108

SUMMARY 113

BIBLIOGRAPHY 116

LIST OF TABLES

Page

I. Trypsin digestion of basement membrane antigens 55

II. Fractionation and purification of streptococcal cell membrane by differential centrifugation 57

III. Immunoblot analysis of solubilized SCM 62

IV. Preparation of monoclonal antibody-coupled affinity column 64

V. Specificity of mAb affinity column 65

VI. Coupling of monoclonal antibody to tresyl-activated column and affinity purification of membrane antigens 70

VII. Plate binding capacity of SCM polypeptides 77

VIII. Efficiency of mAb affinity column binding by individual SCM antigens 83

IX. Affinity constants and valence for the interaction of mAb with SCM antigens 101

X. Comparison of the reactivity of native and denatured SCM antigens 103

XI. Amino acid composition of SCM antigens 105

XII. Summary of amino acid sequencing experiments 108

LIST OF FIGURES

Page

1. Purification of monoclonal antibody from ascites by affinity chromatography 50

2. Sodium dodecyl sulfate electrophoresis of purified mAb ... 52

3. Recovery of mAb activity in the purified mAb preparation . 54

4. Assay for M12 protein in purified SCM 59

5. Rhamnose determination for purified SCM 61

6. Specificity of mAb affinity column 67

7. Affinity purification of membrane antigens 69

8. HPLC profile of SCM tryptic digest 74

9. HPLC purification of SCM peptides 76

10. Reactivity of HPLC-purified SCM polypeptide antigens 79

11. Specificity of mAb in ELISA 81

12. Inhibition of mAb by SCM antigens 87

13. Solid phase C1q does not bind free mAb 89

14. Inhibition of mAb by GBM antigen 91

15. Competitive binding of antigens on affinity column 94

16. Equilibrium dialysis of SCM antigens 98

17. Least squares non-linear regression plots of equilibrium dialysis binding data 100

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
APSGN	acute post-streptococcal glomerulonephritis
ARF	acute rheumatic fever
AS	ankylosing spondylitis
BCA	bicinconinic acid
BM	basement membrane
BSA	bovine serum albumin
CMMW	corrected minimum molecular weight
CNBr	cyanogen bromide
ELISA	enzyme-linked immunosorbant assay
GBM	glomerular basement membrane
h	hour(s)
HLA	human leukocyte antigen
HPLC	high pressure liquid chromatography
HSA	human serum albumin
Ig	immunoglobulin
kd	kilodalton
LBM	lung basement membrane
m	minute(s)
mA	milliamps
mAb	monoclonal antibody
MW	molecular weight
MMW	minimum molecular weight
MR	mole ratio
MSA	mouse serum albumin
NDRI	National Disease Research Interchange
NSAP	nephritis strain-associated protein
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point
RP-HPLC	reverse-phase HPLC
RS	Reiter's syndrome
s	second(s)
SCM	streptococcal cell membrane
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
v	volts

Single letter amino acid symbols

A Ala	L Leu
C Cys	M Met
D Asp	P Pro
E Glu	R Arg
F Phe	S Ser
G Gly	T Thr
H His	V Val
I Ile	Y Tyr
K Lys	

INTRODUCTION

Infection by specific group A streptococci has been linked to the development of autoimmune disease by several investigators (Futcher, 1940; Rammelkamp et al. 1952; Stollerman, 1969; McLaren et al. 1975; Nissenson, 1975). Models have been proposed to explain the role of specific streptococci in autoimmune diseases such as post-streptococcal glomerulonephritis and acute rheumatic fever. One, the immune complex model (Michael et al. 1966), proposes that the immunopathology observed in post-streptococcal glomerulonephritis is initiated by the deposition of immune complexes containing streptococcal antigen in the glomerular basement membrane. This model does not explain the role of specific strains of streptococci in eliciting the autoimmune disease. Moreover, it has been difficult to demonstrate streptococcal antigens in such immune complexes. A contrasting model has been proposed which might be called the molecular mimicry/autoimmune model. This model proposes that antibodies to specific streptococcal antigens are cross-reactive with immunologically related glomerular basement membrane antigens (Markowitz and Lange, 1964; Lange et al. 1985, 1986; Fitzsimons et al. 1987). Experimentally, this model uses monoclonal antibody (mAb) raised against streptococcal membrane antigens and cross-reactive with glomerular basement membrane antigens to induce tissue damage mimicking post-streptococcal glomerular nephritis. Significantly, the animal model uses only mAb against streptococcal cell membrane to induce glomerular lesions; the animals receive no

streptococcal antigen. This mAb shows dose-dependent binding to glomerular basement membrane as well as dose-dependent induction of disease. Therefore, antigenic similarity between the mammalian basement membrane and streptococcal cell membrane must account for mAb cross-reactivity.

This thesis uses a mAb raised against streptococcal cell membrane and cross-reactive with glomerular basement membrane to isolate and characterize streptococcal cell membrane antigens. These antigens are first isolated by either affinity chromatography or by HPLC-gel filtration of digested streptococcal membranes. Immunochemical analysis of the antigens is performed using this mAb. The immunochemical studies include comparison of reactivities with mAb by direct ELISA, competitive inhibition, and affinity chromatography, and analysis of antibody binding affinities and valences for each antigen by equilibrium dialysis. Structural studies of the antigens included amino acid analysis of related antigens as well as comparison of reactivities in native and denaturing conditions.

Analysis of the streptococcal cell membrane antigens using this mAb antibody will yield information about the nature of the streptococcal membrane epitope. These studies therefore will also have implications for the structure of the related glomerular basement membrane antigens.

Post-streptococcal Disease

Incidence and epidemiology

Acute post-streptococcal glomerulonephritis (APSGN) and acute rheumatic fever (ARF) remain common and serious sequelae of streptococcal infection in some parts of the world. Although the incidence of these post-streptococcal diseases has declined and nearly disappeared in Western countries (Gordis, 1985), the incidence of these diseases, particularly life-threatening cases of ARF, remains high in underdeveloped countries (McLaren et al., 1975). In South Africa and other African countries, ARF is the most common cause of acquired heart disease in children; the prevalence among South African children can be as high as 1,920 per 100,000 (McLaren et al., 1975).

The development of APSGN following streptococcal pharyngitis occurs after a well-recognized latent period. Following infection of the skin, the latent period is 21 to 40 days; after infection of the throat, the latent period is only 7 to 15 days (Nissenson et al. 1979). During this latent period one finds low levels of hematuria and albuminuria (Stetson et al. 1955). The clinical disease, referred to as acute nephritic syndrome, is characterized by edema, hematuria, hypertension, and oliguria, but all of these symptoms do not necessarily occur in every patient (Rodriguez-Iturbe, 1984). Acute nephritic syndrome lasts 4 to 7 days, after which the major clinical signs resolve. Although often self-limiting, as many as 25%

of these patients develop acute renal failure, as measured by serum creatinine of > 2 mg/dl (Rodriguez-Iturbe, 1984).

Histologic findings include massive mesangioproliferative glomerulonephritis. The glomerulus is infiltrated by poly-morphonuclear leukocytes (Sorger et al. 1983), and electron-dense deposits are evident in the subepithelial and subendothelial regions of the glomerulus (Lewy et al. 1971; Hinglais et al. 1974; Sorger et al. 1983). The subepithelial deposits are considered characteristic of this disease, though similar deposits are seen in other diseases such as systemic lupus erythematosus and bacterial endocarditis (Rodriguez-Iturbe, 1984). Further studies have demonstrated glomerular deposits of C3, IgG, and IgM in discontinuous, granular patterns (Sorger et al. 1983). Clinical and histologic findings therefore point to a role for the immune system in the development of APSGN.

Etiology of Acute Post-streptococcal Glomerulonephritis

Post-streptococcal diseases became associated with previous infections by group A streptococci, but the incidence of APSGN and ARF following group A infection was shown to be markedly different (Rammelkamp et al., 1952). Nephritis was associated with group A infection of either the skin or throat and the incidence varied widely from year to year, while ARF occurred primarily following throat infections and the incidence remained fairly constant (Rammelkamp et al. 1952). This suggested that only certain strains of group A were associated with APSGN. That certain strains of streptococci were associated with nephritis was recognized as early

as 1917 (Ophuls, 1917), and streptococcal skin infection was first associated with acute glomerulonephritis by Futcher (1940), who described a previous streptococcal skin infection in up to 28 % of acute glomerulonephritis cases. A study of streptococcal infections revealed that infection by M-types 1, 2, 4, 12, 18, 25, 49, 55, and 60 was found to be associated with subsequent acute glomerulonephritis (Stollerman, 1969). Despite many reports that have since confirmed these associations, the mechanism by which streptococcal infection leads to glomerulonephritis and ARF remains unresolved and controversial.

The etiology of APSGN is especially controversial since many mechanisms have been proposed and investigated. Nevertheless, it appears that the disease is immunologically mediated: a consistent latent period exists between the streptococcal infection and the onset of nephritis (Michael et al. 1972; Nissenson et al. 1979); a regular and transient decrease in serum complement occurs during the acute phase of the disease (Gewurz et al. 1968; Kohler and tenBessel, 1969); immunopathological and ultrastructural studies of the kidney demonstrate deposits adjacent to GBM, often containing complement and immunoglobulin (Michael et al. 1972; Sorger et al. 1983).

Models for acute post-streptococcal glomerulonephritis

Serum Sickness and Immune Complex Models for APSGN

The resemblence of this disease to acute serum sickness did not go unrecognized and several serum sickness models for APSGN were

investigated. In these models (Dixon et al. 1961; Germuth, 1953; Wilson and Dixon, 1971) a single and large dose of foreign antigen is injected and the disappearance of the antigen from circulation is followed. The disappearance is slow until the seventh to fourteenth day, when antigen concentration suddenly drops. At this time, immune elimination occurs, but the antibody production leads to immune complex formation, drop in serum complement, and finally deposition of complement and immune complexes in the glomerular basement membrane and mesangium. The role of prolonged exposure to antigen in causing serum sickness was investigated (Wilson and Dixon, 1971), and it was found that rabbits receiving constant amounts of antigen daily varied in their antibody response, but those animals with low to intermediate antibody responses did develop nephritis. These experiments demonstrate the nephritogenicity of immune complexes, but they do not explain the specific role of streptococci in nephritis because they did not utilize nephritogenic streptococci or streptococcal products.

Most immune complex models for APSGN do not identify the role of streptococcal antigens in immune complex formation and subsequent disease. However, the immune complex models do demonstrate the role of the antibody response in the pathogenesis of APSGN. Contributing to these immune complexes are anti-autologous immunoglobulin. Circulating antiimmunoglobulin titers of 1:32 have been found in up to 43% of APSGN patients (Rodriguez-Iturbe, 1984) and even higher titers early in the disease (McIntosh et al. 1979). Glomeruli of 19/22 biopsies from patients had antiimmunoglobulin activity in

eluted fractions (McIntosh et al. 1978) and significant anti-IgG activity could be eluted from a kidney in a fatal case. (Rodriguez-Iturbe et al. 1981).

How the autologous Ig attains significant immunogenicity has been investigated. It was found that autologous IgG could be altered by streptococcal (S. pyogenes) neuraminidase, leading to increased immunogenicity of the IgG when injected into animals (McIntosh et al. 1971, 1972). Immunoglobulin alteration by streptococcal neuraminidase has been demonstrated for organisms isolated from APSGN patients, and it has been shown that the Ig most sensitive to streptococcal neuraminidase was IgM (Mosquera et al. 1985).

Another mechanism by which anti-Ig could be induced was shown to be related to the presence of Fc receptors for IgG in group A streptococci (Kronvall, 1973). The high affinity of these receptors for light kappa chains and for human serum albumin was shown for nephritogenic strains (Vilches and Williams, 1984). These investigators propose that Ig-coated streptococci could lodge in the kidney and present an altered Ig to the immune system.

The significance of these observations regarding anti-Ig activity in APSGN patients' sera is not clear. Because neuraminidase activity is not restricted to streptococci, these observations do not explain the specific role of streptococci in eliciting this disease. Secondly, Fc receptors also occur on other bacteria and on lympho-cytes which, when non-specifically trapped in the glomerulus, could present altered Ig to the immune system. The serum anti-Ig

activity in APSGN patient's sera could be attributed to each of these mechanisms and therefore may not have any significance in the initiation of the disease.

Streptococcal proteins and toxins related to APSGN

Vilches and Williams (1984) proposed that antibody in the glomeruli of APSGN patients could be due to deposition of immune complexes containing the antibody bound to circulating streptococcal antigens. Alternatively, deposition of streptococcal antigen in the kidney could be followed by antibody binding. Another means by which antibody could become localized in the glomerulus is by specific binding to glomerular antigens cross-reactive with streptococcal antigens. Finally, damage to glomeruli due to immune complex or streptococcal antigen binding or toxicity could alter glomerular antigens and induce auto-reactive antibodies. To address these questions, role of specific streptococcal antigens in APSGN has been investigated.

In a model for streptococcal antigen-induced nephritis, M protein antigens were detected in immune complexes in the kidney (Humair et al. 1969; Kantor, 1965; Kaplan, 1965). When acid-extracted M protein type 1 was injected into mice and rats, M protein formed complexes with fibrinogen and deposited in the kidney. Repeated injections of M protein alone or complexed with fibrinogen also were able to induce nephritis in these animals (Humair et al. 1969; Kantor, 1965). The presence of M protein in immune complexes in the kidneys and the nephritogenicity of M protein when injected does not

necessarily implicate M protein in the etiology of APSGN. Second attacks of APSGN are extremely rare (Treser et al. 1971), and it is believed that immunity to the nephritogenic organism is achieved. Since primary exposure to a nephritogenic strain of a particular M type tends to protect from second attacks, and several nephritogenic M types are known, it is possible that these strains express an epitope conserved among their M proteins or express a common non-M antigen. Furthermore, circulating antibody from children with APSGN was able to bind kidney biopsies independently of the M-type with which the children were previously infected (Treser et al. 1971). This suggests that antigens are available in the patients' kidneys that are recognized by the circulating antibody, and this antibody reacts either directly with kidney antigens or with streptococcal antigens other than M protein.

An intracellular streptococcal antigen (Lange et al. 1976) was isolated from the supernatant obtained after centrifugation of pressure-disrupted streptococci. This intracellular antigen could absorb antibodies in convalescing serum, preventing the reactivity of the serum antibodies with antigens in the glomeruli. Purification by column chromatography and isoelectric focusing revealed a protein with molecular weight 43,000 - 50,000 and a pI of 4.7. However, detectable titers to this antigen were shown in 10 - 74% of normal sera. The role of this antigen in the disease process is therefore not clear.

Vogt et al (1983) did chromatofocusing of streptococcal proteins in the supernatant of a defined medium and identified a catio-

nic antigen in the pH range 8-11. This cationic antigen was found in 8/18 renal biopsies of patients with APSGN. All 18 sera had antibody levels to this cationic antigen. This antigen is thought to bind glomerular basement membrane by virtue of its cationic nature.

Villarreal et al. (1979) identified a protein called the nephritis strain-associated protein (NSAP) in the culture supernatant of streptococci grown in Todd-Hewitt broth. The molecular weight and pI were as described above by Lange (1976). Antibodies to this protein were found in serum of 90% of patients with APSGN and in serum of 5 - 15% of patients with ARF, but only 13% of normal sera had antibody activity. That so many patients had antibody activity to this antigen may be because this is a secreted extracellular antigen.

The NSAP from group A streptococci has been identified by Johnston and Zabriskie (1986). Amino acid analysis and sequencing of its 21 amino-terminal amino acids indicate that the secreted extracellular protein is a streptokinase (MW 46 kd). This streptokinase is related to but antigenically different from that of group C streptococci. The streptokinase genes of different strains of group A streptococci were analyzed by Huang et al. (1989) and shown to be extremely heterogenous, although nephritogenic strains appeared to be similar in the hypervariable regions of the gene.

Finally, the nephritis strain-associated protein was shown to be a plasminogen activator and was shown to be directly involved in the development of nephritis (Holm et al. 1988). In a unique tissue

cage model, the investigators were able to demonstrate that localized infection of rabbits with NSAP containing strains of group A streptococci resulted in binding of this protein to the glomerular tissue followed by the development of nephritis in three weeks. Animals infected with NSAP-negative strains did not develop nephritis (Holm et al. 1988). The association of this streptococcal protein with experimentally-induced APSGN may be related to its function as a plasminogen activator. This protein could promote the spread of streptococci through conversion of plasminogen to plasmin, which could cause subsequent degradation of tissue fibrin. Fibrin is often found deposited among the proliferating cells of the glomerulus in patients with APSGN (McCluskey et al. 1966; Feldman et al. 1966). However, how this plasminogen activator is involved in the immunologically-mediated aspects of APSGN is not clear.

Autoimmune Model for APSGN

The immune complex models for the etiology of APSGN do not adequately explain the role of specific streptococci or streptococcal products in the pathogenesis of this disease. Other work with antigens isolated from nephritis-associated strains of streptococci has been either inconclusive or can not explain the immunologically-mediated aspects of APSGN. The autoimmune model for the etiology of APSGN proposes that the immune response to specific kidney antigens results in the immune cascade that leads to the nephritic syndrome in APSGN.

Experimental autoimmune glomerulonephritis has been induced in

animals by administering heterologous mammalian antigens or antibody to related antigens. Sado et al. (1984) showed development of glomerulonephritis in rats that was dose-dependent on the amount of bovine GBM antigen administered. These animals developed lung hemorrhages, proteinuria, hypercholesterolemia, hypertriglyceridemia and hypoalbuminemia with severity directly related to the amount of heterologous antigen injected. However, the linear deposition of IgG and lung hemorrhages suggested that this disease resembled Goodpasture's syndrome or human anti-GBM disease rather than APSGN (Sado et al., 1984).

Another model for autoimmune glomerulonephritis showed that mAb prepared against collagenase digested glomeruli caused massive transient proteinuria in rats (Orikasa et al., 1988). The mAb localized to the slit diaphragms of glomerular foot processes which are responsible for the integrity of GBM and its filtration capacity. These results suggest a mechanism for inducing the nephritic syndrome observed in APSGN and show the autoimmune potential of anti-kidney antibody. However, autoimmune models for APSGN must take into account the specific role of streptococci.

The autoimmune model more specifically defined as "molecular mimicry" takes into account the streptococcal basis for the disease. This model predicts that a specific antigen exists in nephritogenic streptococci that is immunologically similar to an antigen in the kidney. The molecular mimicry model would explain why specific strains of streptococci are associated with the disease. It would further explain why certain individuals are susceptible to this dis-

ease: only those individuals who express the autoantigen would suffer the immunopathologic consequences of antibodies to the specific streptococcal antigens.

Early studies suggested that low molecular weight streptococcal products were related to APSGN and that antibody to these products could cause nephritic syndrome reminiscent of APSGN. Kelly and Winn (1958) were able to induce a nephritic syndrome by implanting dialysis bags containing live nephritogenic streptococci intraperitoneally in rabbits. These observations indicate that a specific dialyzable substance from streptococci was responsible for the nephritic syndrome, though the mechanism by which this factor caused nephritis remained unclear. In 1959, Matheson and Reed described a nephritogenic factor from a group A streptococcus isolated from children with APSGN. Rabbit antisera to this factor was able to induce nephritic syndrome in rabbits, including hypertension, albuminemia, and hematuria (Matheson and Reed, 1959). Antisera to other streptococcal fractions could not cause these symptoms. This factor was found in cell-free culture filtrates and was found to be dialyzable (< 10,000 MW). Further characterization indicated that the antigen was neither carbohydrate nor lipid. The < 10,000 MW factor was purified by ammonium sulfate precipitation and the most nephritogenic material was found to be precipitated between 65 and 70% saturation. Thirteen of fifteen ninhydrin spots could be identified in paper chromatography as specific amino acids. Further characterization of the purified material by paper electrophoresis indicated that 6 distinct components could be identified in this

fraction on the basis of their migration properties. Based upon these findings, these investigators propose that this factor which is able to produce nephritogenic antisera in rabbits is a single polypeptide or group of polypeptides of less than 10,000 daltons. A factor with similar chemical and physical properties could be isolated from non-group A streptococci, but this material did not induce nephritogenic antisera in rabbits. The polypeptide antigen could not be derived from washed, heat-killed group A cells, but was found only in supernatants of growing cells, suggesting that it is a secreted intracellular or membrane-associated antigen (Matheson and Reed, 1959). These results support the autoimmune model for APSGN because a discrete streptococcal antigen could induce nephritogenic antibodies.

Markowitz (1960) proposed an immunologic relationship between mammalian glomerular basement membrane (GBM) and group A type 12 beta-hemolytic streptococci. Markowitz showed that injection of this strain of streptococci into the peritoneal cavity of rats induced extensive kidney lesions after four weeks. This delayed onset of kidney pathology suggested that the immune response to the streptococcal infection was responsible for the kidney disease. Following these observations, Markowitz and Lange (1964) showed that immunization of animals with the streptococcal cytoplasmic membrane (SCM) induced the formation of antibodies that reacted directly with human glomerular basement membrane antigens. Thus the anti-streptococcal cell membrane antisera was potentially nephritogenic. Anti-SCM serum has also been shown to have an effect on host

tolerance to homografts (Rapaport et al. 1969). Rabbit anti-SCM antisera caused accelerated skin homograft rejection in a high percentage of guinea pigs receiving the antisera prior to the graft, indicating reactivity of this serum may extend to skin membrane. Together, these observations are significant because they point to the autoimmune potential of the anti-SCM antisera.

Other work further supports the role of the streptococcal cell membrane (SCM) in the autoimmune model for APSGN. Treser et al. (1970) showed that a component of the plasma membrane derived from group A type 12 streptococci could absorb IgG from the sera of APSGN patients and thus prevent reactivity with GBM and glomerular mesangium. Only group A abolished reactivity with the kidney sections as measured by immunofluorescence. Sucrose density-gradient ultracentrifugation of streptococcal fractions localized the antigens to the membrane and identified two possible antigens. One was soluble in phosphate-buffered saline and the other was insoluble. The insoluble fraction was a lipoprotein with MW of approximately 120,000. Significantly, M protein, cell wall, and carbohydrate derived from this group A type 12 organsism were unable to absorb autoantibody activity (Treser et al. 1970).

Other work utilizing immunofluorescence has supported these findings. Antisera raised against whole basement membrane, or a constituent thereof, produce a linear pattern of staining when evaluated by indirect fluorescent antibody techniques. In post-streptococcal glomerulonephritis, however, one sees a granular subepithelial deposition of IgG and complement in the GBM. Using

indirect immunofluorescent staining procedures, Blue and Lange (1975) showed that rabbit anti-SCM antibodies bind human GBM in a granular pattern. This suggests that the anti-SCM antibodies are cross-reactive with distinct spatially defined epitopes in GBM. Furthermore, this binding was inhibited if the antisera was pre-absorbed with either SCM or GBM.

High levels of anti-SCM antibodies in the sera of APSGN patients were reported by Yoshimoto et al. (1987). Only serum from APSGN patients could bind Triton-X 100 extracted SCM antigens in ELISA and SDS-PAGE isolated antigens in Western blots. At least four antigens were detected by Western blotting (though the authors do not state the antigen molecular weights they appear to be approximately 67-70, 40, 25, and 20-22 kd). Four patients reported had antibodies only to the largest antigen, while another patient had antibodies only to the three smallest antigens, indicating either infection by different streptococcal serotypes or genetic differences in antibody responses. Unfortunately, the serotype of the streptococci with which the patients were infected was not known, but the SCM used by these investigators was prepared from group A type 12 (Yoshimoto et al. 1987).

In summary, evidence from work with different animals and with APSGN patients has shown that antibodies to SCM are cross-reactive with kidney tissue. Furthermore, antibodies to SCM can cause accelerated graft rejection and APSGN-like disease in animals, indicating that the auto-reactive nature of these antibodies, and not SCM antigen itself, is the key to the disease. A critical step in

the autoimmune model for APSGN was the development of a simple and controlled animal model that demonstrated the autoimmune potential of anti-SCM antibodies.

An animal model for APSGN was developed following the observations by Lange et al (1985) who showed that rabbit anti-SCM could induce glomerulonephritis in mice, and could also induce synthesis of murine GBM, a pathology mimicking that seen in the human disease. Since this work used heterologous antibody to induce the disease in mice, it was possible that the resulting pathology was due to immune complexes resulting from species-differences in the immunoglobulin administered and not necessarily to the autoreactivity of the anti-SCM antiserum. Homologous antibody with defined specificity was obtained when hybridomas from SCM-immunized mice were produced by Lange and Weber (1985). Antibodies specific for GBM and SCM were detected by ELISA using plates coated with trypsin or collagenase-digested GBM. Several monoclonal antibodies were produced (Lange and Weber, 1986) that were highly cross-reactive with GBM and SCM. When these clones were propogated intraperitoneally in mice, the animals developed hemorrhagic lungs as well as kidney lesions, indicating the ability of these antibodies to react with basement membrane other than GBM. The pathology was dose-dependent and greater in females than in males. Fitzsimons et al (1986, 1987) confirmed reactivity with lung by showing mAb binding to isolated soluble lung basement membrane in ELISA.

Characterization of streptococcal cell membrane

The pathologic nature of anti-SCM mAb having been established, the isolation and characterization of the nephritogenic SCM antigens became possible employing these mAbs. Recent work has focused on the characterization of SCM antigens rather than the host autoantigens for the following reasons: because of the cross-reactivity of these mAbs with GBM and LBM, information regarding the nature of the SCM antigen should have important implications for the immunologically related mammalian antigens; SCM is chemically well-defined, thus analysis of the relatively simple streptococcal cell membrane would seem a much easier task than analysis of the complex heterogeneous basement membrane; furthermore, SCM is available in large amounts and easily isolated in a pure form; finally, SCM from a known nephritogenic strain can be employed, whereas basement membrane may have individual variations in autoantigen expression or uncontrolled and unknown histories.

Chemical composition of streptococcal cell membrane

The chemical characterization of streptococcal cell membranes has been the subject of a limited number of investigations. SCM has been isolated by a variety of methods, including mechanical disruption of whole cells by glass beads (Markowitz and Lange, 1964) or Mickle disintegrator (Freimer, 1963), which result in fragmented membrane, and degradation of group A cell wall by streptococcal group C phage-associated lysin, which results in wall-free protoplasts (Kessler and van de Rijn, 1979; van de Rijn and Kessler,

1979; Freimer, 1963). Whatever the method of SCM isolation, its reported chemical composition varies little.

Freimer (1963) reported that total protein for SCM isolated by lysis with the phage-associated lysin was 68% and SCM obtained by mechanical disruption in a Mickle disintegrator was 66.9%. In another report, SCM obtained by disruption of whole heat-killed cells with glass beads was 80% protein (Markowitz and Lange, 1964). Van de Rijn and Kessler (1979) reported that protein was 70-73% of the dry weight of membranes isolated from whole cells by phage-associated lysin. Though the total protein remained the same regardless of the growth phase of the cells, van de Rijn and Kessler showed that the level of expression of certain membrane polypeptides was altered depending on whether the cells were in exponential or stationary phase growth. Several proteins were increased during exponential phase, most of which were peripheral membrane proteins, though some integral proteins were increased in stationary phase (van de Rijn and Kessler, 1979).

The total lipid composition for membranes ranges from 15-25% for SCM from protoplasts and disrupted cells (Freimer, 1963; van de Rijn and Kessler, 1979). Total carbohydrate, most of which is glucose, is usually 2% of the membrane weight. Rhamnose is found in trace amounts, and less than 0.1% is characteristic of wall-free membrane (Freimer, 1963). Other carbohydrates are all simple sugars in trace amounts, including galactose, glucosamine, galactosamine, ribose; no sialic acid and only 0.2% hexosamines have been reported (Markowitz and Lange, 1964). Phosphorous is 1-3% of the membrane

(Freimer, 1963; Markowitz and Lange, 1964; van de Rijn and Kessler, 1979).

Amino acid analyses by different investigators show consistent results. Different investigators have found high concentrations of glutamic and aspartic acids (Freimer, 1963; Markowitz and Lange, 1964; van de Rijn and Kessler, 1979; Agostino and Lange, 1985).

Immunochemical characteristics of streptococcal cell membrane

Most work has revealed protein or glycoprotein as the major antigenic component of SCM. Using whole rabbit anti-SCM antisera, SCM from types 12 and 25 group A streptococci was immunochemically characterized (Freimer, 1963). SCM from protoplasts was extracted with ethyl ether and ethanol and then digested with either trypsin, chymotrypsin, or papain. About 50% of the membrane protein was estimated to be solubilized by this treatment. Immunoelectrophoresis in agar of the trypsin-soluble SCM vs. rabbit anti-SCM sera showed 2 overlapping precipitin arcs which migrated toward the cathode (Freimer, 1963). Capillary precipitin assays also showed antigens in SCM boiled 10 m at pH 2.0. How this material was related to the enzyme-digested antigens was not clear.

SCM obtained by disruption of type 12 whole cells was extracted with the fluorocarbon trifluorotrichloroethane and the soluble fraction from the aqueous phase was digested with trypsin (Markowitz and Lange, 1964). This material was immunochemically characterized by immunodiffusion, passive cutaneous anaphylaxis, and passive hemagglutination. Rabbit antisera against this SCM fraction was

found to be cross-reactive with human glomerular basement membrane by each of these methods. The soluble SCM fraction was determined to be 8.5 kd by ultracentrifugation, amino acid analysis, and chromatography with Sephadex (Markowitz and Lange, 1964). Electrophoretic analysis showed that the SCM antigen contained one component which migrated toward the anode, and possibly two toward the cathode. This SCM fraction which showed immunologic similarity with GBM was further chemically characterized by Lange and Markowitz (1969). Digestion of this fraction with papain and peptide mapping with high voltage electrophoresis yielded a peptide fingerprint very similar to that obtained for GBM. This cross-reactive material was found in SCM extracted with SDS, CNBr, or deoxycholate, but not with TCA, pancreatic lipase, Triton X-100, 2-chloroethanol, pepsin, or collagenase (Lange, 1969). DEAE cellulose chromatography of each soluble preparation yielded one fraction eluted with 0.01 M phosphate, pH 7.75, which was cross-reactive in agar gel analysis with GBM (Lange, 1969). These results gave the first immunochemical data for SCM antigens which are immunologically related to GBM.

The role of glycoprotein carbohydrate in the reactivity of SCM and GBM antigens with anti-SCM antisera has been examined (Blue and Lange, 1975, 1976a, 1976b; Blue et al. 1980). Indirect immunofluorescence demonstrated that anti-SCM antisera binding to GBM increased when the GBM was pretreated with carbohydrase (Blue and Lange, 1975), indicating reactivity of the antisera with a protein epitope. Thus, the carbohydrates removed by treatment with carbohydrase were thought to "mask" the dominant protein epitopes in

GBM. When antisera was prepared to "carbohydrate-rich" SCM, this antisera showed no cross-reactivity with GBM (Blue et al. 1980). It was concluded that the epitope in SCM and GBM responsible for the antisera cross-reactivity was protein.

Specific aims of present work

Guiding this investigation is the hypothesis that the observed cross-reactivity of anti-SCM antisera and mAb with GBM is due to molecular similarities between these antigens. Therefore immunochemical characterization of SCM antigens using an anti-SCM mAb cross-reactive with GBM should have implications for the GBM antigen.

The aims of this work are to isolate streptococcal cell membrane protein antigens immunologically related to glomerular basement membrane, to characterize the interaction of the antigens with the mAb and to identify minimal structural requirements for reactivity of the SCM antigens with the mAb. Isolation of SCM antigens is accomplished with anti-SCM mAb affinity columns and by HPLC gel filtration. The reactivities of the antigens recovered are then analyzed and compared by ELISA, competitive inhibition, and affinity chromatography. Interaction of the recovered antigens with mAb is further characterized by equilibrium dialysis. Analysis of the minimal structural requirements for antigenicity is attempted by amino acid analysis and sequencing of purified antigens and a study of their reactivity with mAb in native and denaturing conditions.

Monoclonal antibody

Spleen cells from mice (6 wk old Balb/c) immunized with particulate SCM in complete Freund's adjuvant were fused with P3-X63-Ag8 nonsecretor plasmacytoma cells as described by Fitzsimons et al. (1987) using the procedure of Goding (1980). Clones showing reactivity to both SCM and GBM by ELISA were selected by limiting dilution three times to insure monoclonality. Ascites was prepared from the reactive clones as descibed by Fitzsimons et al. (1987) and the ascites and serum were employed as the source of mAb. Ascites from non-immune animals or animals carrying the fusion partner alone did not exhibit anti-SCM activity (Fitzsimons et al. 1987).

Determination of monoclonal antibody isotypes

Isotyping was performed with monoclonal anti-mouse isotype antisera (Hyclone, Logan, UT.) according to the manufacturer's directions. Briefly, goat anti-mouse isotype specific (γ_1, γ_{2a}, γ_{2b}, γ_3, α_1, α_2, μ) antibodies were coated (100 ul/well) on ELISA plates (Falcon) for 1 h at 22°C. The plates were washed and 100 ul ascites was added to each well. After 1 h at 22°C, the wells were washed and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin was added to each well to detect the captured ascites immunoglobulin. After incubation for 1 h the plates were washed and 150 ul p-nitrophenol phosphate (20 ug/ml) was added to each well and the amber color allowed to develop. The reaction was stopped by

addition of 50 ul 0.1N HCl to each well.

Affinity purification of mAb from ascites

SCM tryptic digests were coupled to two different gel media to affinity purify mAb from ascites. The first column was made using cyanogen bromide-activated Sepharose. Sepharose gel was first swelled in 0.001 M HCl on a glass filter and then washed with distilled water. The slurry was added to 100 ml 2 M sodium carbonate and mixed thoroughly. To 50 ml packed volume gel was added 5 ml cyanogen bromide solution (cyanogen bromide 2 gm/ml in 100% acetonitrile) with vigorous stirring for 2 m. The gel was poured into a coarse sintered-glass funnel under vacuum and washed with 300 ml 0.1 M sodium bicarbonate, pH 9.5, 300 ml deionized water, followed by 300 ml 0.2 M sodium bicarbonate, pH 9.5. The gel then was suctioned until it was a firm moist cake and then transferred to a bottle with 50 ml 0.2 M sodium bicarbonate, pH 9.5. The antigen was added with constant gentle stirring at 4^{o}C for 20 h. After coupling, 5 ml 1 M glycine was added to block reactive sites and mixed for 4 h at 4^{o}C. The gel then was transferred to a sintered-glass funnel and washed with 20 volumes (1 l) 0.1 M sodium acetate, pH 4.0, 1 liter 2 M urea, 1 l 0.1 M sodium bicarbonate, pH 10. The gel was reconstituted in 50 ml PBS, degassed, and used immediately. The second column was a carbonyldiimidazole-activated support (Reactigel, Pierce Chemical Company, Rockford, IL). The pre-activated gel (15 ml final volume) was coupled with the antigen in PBS, pH 8.5, for 30 h at 4^{o}C. The gel required no blocking and was used after washing

in a sintered-glass funnel with PBS.

The efficiency of antigen coupling to the gels was monitored by taking samples of the supernatants at 4 h and at the end of the coupling time period. The supernatants were assayed for protein content using the BCA method (see "Protein determinations") which showed that 4.7 mg SCM was bound per ml of the Reactigel media and 3.0 mg SCM was bound per ml of the CNBr-activated Sepharose. HPLC analysis of the supernatants indicated that the gels bound most of each peptide in the SCM digest (11.6, 9.2, 7.0, 4.7, 2.3 kd), but the Reactigel did not efficiently bind the 4.7 kd and the CNBr-Sepharose did not bind the 9.2 kd.

To each 1 x 10 cm column were added samples of ascites (200, 100, 50 ul) in PBS. The columns were washed with PBS 1 drop/15 s. The effluents were collected and the columns were washed with 0.1 M glycine-HCL, pH 2.0, at a flow rate of 1 drop/6 s. The eluent was collected in PBS, pH 8.0, and then neutralized with 0.1 N NaOH. The presence of mAb activity in these effluents and eluates was evaluated by ELISA using SCM-coated plates.

Euglobulin precipitation of IgM mAb

IgM mAb was purified by euglobulin precipitation (Garcia-Gonzalez et al. 1988) from ascites fluid and pooled serum of mice carrying one particular clone. Following dialysis against two changes distilled water for 20 h at 4°C, the recovered IgM-rich precipitate was solubilized in borate buffered saline, pH 8.4.

IgM content was determined by serial dilutions employing ELISA

plates coated with goat anti-mouse IgM (Hyclone, Logan, UT). ELISA plates were coated with goat anti-mouse IgM 6.0 ug/ml in PBS, pH 7.4, for 1 h at 22°C. The plates were washed 15 times with PBS containing 0.03 % Tween-20 (PBS-Tween). The IgM-rich preparation, the starting ascites, and the supernatants of the precipitations were added to the wells (100 ul/well) at various dilutions (1:100 to 1:-10^6). TEPC, a BALC/C myeloma IgM, was added to separate wells at various dilutions for direct comparison and determination of IgM content in the various preparations. After incubation for 1 h at 22°C, the wells were washed 15 times with PBS-Tween and to each well was added 100 ul horseradish peroxidase-conjugated goat anti-mouse immunoglobulin, 0.2 ug/ml in PBS. The plates were incubated for 1 h at 22°C and washed 15 times. The substrate was added as described in "Enzyme-linked immunosorbant assays" and the color development measured at 405 nm as described. The absorbance was plotted vs. the inverse dilution of mAb. The concentration of IgM mAb was calculated from three points in the linear portion of the curve and by multiplying the mAb dilution factor by the known TEPC concentration at each absorbance. The average value was 2.21 mg/ml; protein determination using the bicinconinic acid protein assay gave a value of 2.10 mg/ml.

Purity of the precipitated mAb was assessed by cellulose acetate electrophoresis and sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). The recovery of IgM mAb activity was tested in ELISA using trypsin-digested SCM as the solid phase antigen.

Isolation of basement membrane antigens

Human renal cortex and lung were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Mice were Balb/c 1-6 months old, and were housed in the Animal Research Facility of Loyola University Stritch School of Medicine.

Purified BM was prepared by the procedure of Carlson et al. (1978) and as described by Lange (1980a). All solutions were kept cold and work was done on ice. Murine kidney was pressed gently through a 200 mesh screen; human kidney or lung was similarly processed using 100 mesh screen. The screen was washed with phosphate-buffered saline (PBS), pH 7.4, and the remaining tissue was pushed through. Processed tissue was collected in a watch glass and placed in a 50 ml conical centrifuge tube filled with PBS containing tannic acid (tPBS, 50 mg tannic acid/l PBS). The tubes were shaken well and the contents allowed to settle for 30 m. After 30 m, 2/3 of each tube was aspirated. The tubes were refilled with tPBS and the contents allowed to settle for 30 m. This settling was repeated 3 times. The tubes then were centrifuged 10 m at 700xg. The PBS supernatants were aspirated and replaced with 8 ml H_2O (0.05% sodium azide). The contents were allowed to settle for 2 h at 22°C. The tubes then were centrifuged 10 m at 700xg. The supernatants were aspirated and the sediments resuspended in 3% Triton X-100. The contents were allowed to settle at 22°C for 2-4 h. Following centrifugation and aspiration of the supernatant, deoxyribonuclease (0.001 % in 1.0 M NaCl, Sigma, St. Louis, MO) was added and the sediment was resuspended and incubated for 1-2 h at 37°C. The tubes

then were centrifuged and the supernatants aspirated. To each tube was added 4% sodium deoxycholate in 0.05% sodium azide. After 20 m at 22°C the tubes were centrifuged and the supernatants removed. The sediment were washed with 0.05% sodium azide 5 times. The clear supernatant was aspirated, and the sediment was frozen at -4°C. Microscopic examination of this sediment confirmed the presence of glomeruli free from cellular debris. This observation is consistent with the results obtained by Carlson et al. (1978) and Lange (1980a).

Purified BM was digested with trypsin (Sigma, St. Louis, MO). A 1 mg/ml trypsin solution (0.1 M NH_4HCO_3 0.1 mM $CaCl_2$, pH 8.0) was added to BM (1 mg trypsin/100 mg BM) and tubes were rotated for 20 h at 37oC. The undigested material was removed by centrifugation. The supernatants of these enzyme digestions were dialyzed against several changes of distilled H_2O at 4°C and then concentrated by vacuum centrifugation. The soluble protein content was determined by the BCA method (see section titled "Protein determinations").

Isolation of streptococcal membrane antigens

The strain employed exclusively was group A M-type 12 S.pyogenes Hektoen strain (Markowitz and Lange, 1964). Whole cells of heat killed Type 12 streptococci were washed in saline repeatedly at 13,000xg until the supernatants measured < 0.2 absorbance units at 278 nm. The cells were disrupted with the Bead Beater cell disrupter (Biospec Products) using 0.1 mm diameter glass beads previously washed with 50% sulfuric acid followed by several washes

with distilled water. The chamber was half filled with beads and then approximately 100 gm wet weight whole cells were added. The chamber was filled to the mouth with water to exclude air and then surrounded with ice. The cells were disrupted by five cycles of 5 m of disruption followed by 5 m of rest.

Differential centrifugation with a continuous flow head in a Sorvall refrigerated centrifuge separated whole cells and wall material from membrane (see Results). Pelleted wall and membrane was dialyzed and frozen or lyophilized. Rhamnose determination of the membrane confirmed its purity (described in detail below). Contamination with wall-derived M protein was assessed by inhibition ELISA: SCM was added to anti-M 12 antisera which was then assayed for reactivity on M 12-coated ELISA plates.

Rhamnose content of streptococcal cell membrane

The procedure of Dische and Shettles (1948) was used to determine the rhamnose (methyl pentose) level in the membrane preparation. A level of < 0.1% is acceptable for wall-free streptococcal cell membrane (Freimer, 1963). To ice cold standard solutions of methyl pentose (2.75, 5.50, 8.25, 10.0 ug/ml in 1 ml distilled water) were added 4.5 ml chilled sulfuric acid (6 parts concentrated sulfuric acid:1 part deionized water) slowly, with constant shaking in an ice bath. After standing for 10 m at 22^{o}C, the tubes were placed in a boiling water bath for 3 m. The tubes were allowed to stand several m at 22^{o}C. To each tube was added 0.1 ml 3% cysteine hydrochloride. The contents were mixed, and after 2 h the ab-

sorption at 396 nm and 430 nm was measured. Subtraction of the absorbance at 430 from the absorbance at 396 corrects for all absorbance except that due to methyl pentose. The SCM samples were first digested in concentrated sulfuric acid and boiled for 3 m. They then were used in the assay. The % rhamnose was determined by examining the standard curve and assigning a concentration to the unknown.

Preparation of soluble SCM antigens

SCM was solubilized with trypsin or with SDS. Trypsin digestion was as described for BM antigens. SCM was solubilized with SDS by mixing 20 mg SCM with 1% SDS in PBS for 1 h at 22°C. The SDS-extracted SCM was centrifuged 14,000xg 10 min to remove particulate material. The supernatant was dialyzed at 4°C for 48 h against several changes 0.1 M KCl, 0.05% NaN_3. The supernatant was collected and dialyzed against distilled H_2O 4 h at 4°C. The supernatant was collected and concentrated. The concentrated extract was passed over a 10 cm column of Biobeads SM2 adsorbant (Biorad) equilibrated in 10 mM KPO_4, pH 7.2. One ml extract was layered on the column and the column was washed with 10 ml 10 mM KPO_4. Fractions were collected and assayed for protein by BCA as described in "Protein determinations". The column was regenerated by washing with 4 bed volumes distilled H_2O followed by 4 bed volumes methanol, finishing with 4 bed volumes of distilled H_2O.

Both SDS-extracted and trypsin-digested SCM were screened for soluble antigens as described in "Immunoblot of SCM antigens".

Type-specific M-12 proteins

Type-specific M-12 protein was obtained by hot-HCl extraction and purified as described by Lange et al. (1969). Cell walls were stirred with hot concentrated HCl. Insoluble material was removed by centrifugation. The supernatant was neutralized with 6 N NaOH and the crude M protein extract was fractionated by ammonium sulfate precipitation between 30 and 60 % saturation. After dialysis and lyophilization, samples were dissolved in phosphate buffer 0.005 M, pH 7.75, and chromatographed on diethylaminoethyl cellulose columns. Elution was stepwise with buffers of increasing ionic strength and decreasing pH. Individual peaks were collected and rechromatographed on carboxymethyl cellulose equilibrated with 0.01 M, pH 2.5 glycine buffer.

C1q purification

C1q was purified from human serum by three cycles of precipitation at low ionic strength as described by Yonemasu and Stroud (1971). Fresh serum (240 ml) was dialyzed with stirring for 18 h at 4°C against 4 l of the following solution: 0.01 M EDTA, 0.005 M NaN_3, 0.0005 M phenylmethylsulfonylfluoride, pH 6.0. The serum was centrifuged to remove residual red cells and clots. The serum was then dialyzed with stirring against 4 l 0.026 M EGTA, 0.03 M NaCl, pH 7.5 for 4 h at 4°C. The dialysate was changed and dialysis continued in the same buffer for 15 h at 4°C. The precipitate was collected by centrifugation and washed in the dialysis buffer. The precipitate was redissolved in 25 ml of 0.75 M NaCl, 0.01 M EDTA,

0.02 M acetate, pH 5.0. Aggregated material was removed by centrifugation. The redissolved precipitate was then dialyzed with stirring against 4 l of 0.06 M EDTA 0.065 M NaCl, pH 5.0 for 4 h at 4°C. The precipitate was recovered by centrifugation and washed in the same fresh dialysis buffer. The precipitate was redissolved in 25 ml 0.75 M NaCl 0.01 M EDTA 0.005 M NaH_3HPO_4, pH 7.5. Aggregates were removed by centrifugation. The redissolved precipitate was then dialyzed with stirring against 4 l of 0.0035 M EDTA 0.069 M NaCl, pH 7.5 for 4 h at 4°C. The precipitate was collected by centrifugation and washed by resuspension and centrifugation in the same fresh dialysis buffer. The precipitate was redissolved in 1.0 ml 0.75 M NaCl 0.01 M EDTA 0.02 M acetate, pH 7.5. The protein content was determined by BCA as described in "Protein determinations". The purity was confirmed by cellulose acetate electrophoresis (see "Cellulose Acetate Electrophoresis") of the starting serum, the supernatants of the first, second, and third precipitations, and the material obtained in the third precipitation.

C1q was diluted in high-salt PBS-EDTA (0.64 M NaCl 0.01 M EDTA, pH 7.8) to a concentration of 25 ug/ml and coated 100 ul/well on 96-well ELISA plates for 1 h at 22°C. The wells were emptied and the supernatants saved. Uncoated sites in the wells were blocked by the addition of 1 % BSA in PBS (0.01 M EDTA) 100 ul/well at 22°C for 2 h.

Protein determination

Protein concentrations were determined by the bicinchoninic

acid assay as described by Smith et al. (1985) (Pierce Chemical Company, Rockford, IL). To each well of an ELISA plate (Falcon) was added 10 ul of each sample with 200 ul BCA working reagent. Standard concentrations of bovine serum albumin (Pierce) were added to separate wells of the same plate. A blank consisting of 10 ul solvent and 200 ul BCA working reagent was prepared. The plate was covered and incubated 30 min at 37°C. The blue-green color was read at 570 nm in a Dynatech (Alexandria, VA) model 500 ELISA reader. Comparison of unknowns to the standard curve permitted determination of protein concentrations. This assay is sensitive to protein concentrations in the range of 10 to 1200 ug/ml (Smith et al. 1985).

High pressure liquid chromatography

The HPLC system includes the Waters M-45 solvent delivery system and model 440 absorbance detector (Millipore, Bedford, Mass.). The columns used were the Waters I-125 and the DuPont GF-250 (DuPont, Wilmington, Delaware), both gel filtration columns. The I-125 (7.8 mm x 30 cm) is composed of a bonded silica support and is able to separate proteins of 2-80 kd. The silica based packing is compatible with aqueous solutions and separates by size proteins with acidic, neutral, and mildly basic pIs. The GF-250 (9.4 mm x 25 cm) is a zirconia-stabilized silica support capable of separating proteins of 2-250 kd.

All solvents used in HPLC were filtered before use with a 0.45 um pore size 44 mm filter (American Scientific Products, McGraw

Park, Il.). All samples were centrifuged to remove particulates and then filtered before injection. The I-125 was run in 0.05 M NaH_2PO_4 pH 7.8 at 2 ml/min and the GF-250 was run in 0.1 M Na_2HPO_4 pH 6.0 at 1 ml/min.

A standard curve was prepared using the HPLC Molecular Weight Marker Kit (United States Biochemical Corp., Cleveland, OH.), which contains glutamate dehydrogenase (290 kd), lactate dehydrogenase (140 kd), enolase (67 kd), adenylate kinase (32 kd), and cytochrome c (12.4 kd). The molecular weight markers from Sigma were also used. These were bovine serum albumin (66 kd), carbonic anhydrase (29 kd), cytochrome c (12.4 kd), and aprotinin (6.5 kd). The column was run with 0.1 M Na_2HPO_4 pH 7.2-7.4. The average retention time of several runs was used to make a standard curve by plotting log molecular weight vs. the retention time of the protein in m. Molecular weights of unknown samples were determined from the standard curve.

Reverse-phase HPLC was accomplished using the Varian HPLC system. Gradients of 0.1% TFA to 60% acetonitrile in 0.1% TFA were employed with C18, C8, and C4 reverse-phase columns.

HPLC-affinity chromatography

Coupling of IgM mAb to tresyl-activated columns

IgM mAb purified by euglobulin precipitation from ascites and serum was passed over the tresyl-activated silica-based columns (5 cm x 1 cm, Pierce Chemical Company, Rockford, IL). Three individual affinity columns, one for each membrane digest, were activated by pumping through 7 ml mAb (at 5 mg/ml in 0.2 M phosphate buffer, 0.5

M NaCl, pH 7.4) at 1 ml/m. The amount of mAb loaded was determined as described in "Monoclonal antibody"; the amount of mAb in the effluents was determined from the absorption at 278 nm of the effluent and the absorbance coefficient for IgM (1.3 for a 0.1% solution). From these were calculated the amount of IgM bound to the column. The column effluents were recycled and the effluents monitored until it was determined that the columns were not binding additional mAb.

Affinity isolation of membrane antigens

Membrane preparations were loaded onto their respective mAb affinity columns in running buffer (0.1 M Na_2HPO_4, pH 7.8). The columns were washed with three volumes of running buffer, the effluents collected, and the specifically bound antigens were eluted with 5 ml 1 M acetic acid, pH 2.0. The specifically eluted antigen was collected and neutralized with 0.1 N NaOH. Each eluate was analyzed by HPLC gel filtration. Eluted material was concentrated to the volume in which the sample was loaded and the protein concentration was determined by BCA. The concentration of eluted antigen was also determined by comparing the areas of the elution curves to the eluent curves by planimetry. Antigen:antibody nmole ratios were determined from the amount of antigen eluted and the nmoles of mAb (calculated based ug amount bound and molecular size of 800 kd) bound to the matrix.

Specificity of the mAb affinity column

The specificity of the mAb column was assessed by loading related and unrelated proteins on the column. Bovine serum albumin (BSA), M-types 12 and 48 streptococcal M protein and mouse serum albumin each were loaded on the column and the effluents and eluates collected. The amount of protein loaded, in the effluent, and in the eluate was measured by BCA and by monitoring the absorption of each at 278 nm. Amino acid analysis was performed on the M48 eluate to assess whether low amounts of protein were binding to the affinity column. The M48 eluate amino acid composition was compared to that of various buffer and background controls and acid washes. Finally, M12 protein was loaded on the column and the effluent and eluate collected, concentrated, and resuspended in the same volume in which M12 was loaded. These were coated on ELISA plates and assayed for the presence of M12 with rabbit anti-M12 antibody.

Electrophoresis

Cellulose acetate electrophoresis

Separaphore cellulose acetate membranes (5 x 15 cm, Gelman Sciences) were wetted in running buffer (0.075 M barbital buffer, pH 8.6) and samples applied with the Beckman sample applicator. Membranes were loaded into the Beckman Model R-101 microelectrophoresis chamber. Electrophoresis was run 20 or 30 m at 60 v. Membranes were stained with 0.2% Ponceau S (in 3.0% trichloroacetic acid) 10 m and destained in 5.0 % acetic acid.

Sodium dodecylsulfate polyacrylamide electrophoresis

SDS-PAGE analysis was accomplished essentially by the procedure of Laemmli (1970). The running gel contained 10% w/v acrylamide and 0.1% w/v SDS in 0.375 M Tris-HCl buffer, pH 8.8. The stacking gel contained 3.0% w/v acrylamide and 0.1% SDS in 0.125 Tris-HCl buffer, pH 6.8. Samples were boiled 1.5 m in sample buffer which consisted of 2.0% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol (Eastman), and 0.001% w/v bromophenol blue in 0.0625 M Tris-HCl buffer, pH 6.8. To initiate polymerization, 0.025 % tetramethylethylenediamine (Eastman) and ammonium persulfate (Fisher) were added to the gels. Slab gels (1 mm thick) were poured between glass plates 15.9 x 14 cm and allowed to polymerize. The upper tank buffer contained 0.1% SDS in Tris-glycine buffer, (0.025 M Tris, 0.192 M glycine), pH 8.7. The lower tank buffer contained 0.5 M Tris-HCl, pH 8.1. Electrophoresis was carried out at 30 mA/gel for 2 hs. Molecular weight standards (Sigma) were included with each gel. These contained bovine serum albumin, carbonic anhydrase, phosphorylase b, lysozyme, soybean trypsin inhibitor.

Gels were fixed and stained with Coomassie brilliant blue (BioRad). Coomassie staining was accomplished with 0.1% Coomassie in 7.0% trichloroacetic acid. Destaining was done with 25% methanol, 10% acetic acid in water.

Immunoblot and Western blot of soluble SCM antigens

Following electrophoresis, SDS-PAGE gels were equilibrated in electrotransfer buffer (0.1 M 3-[cyclohexylamino]-1-propanesulfonic

acid, pH 11.0, Sigma) for 10 m. PVDF membrane (Millipore) was cut to the size of the gel (approximately 9 x 12 cm) and wetted 5 s in 100% methanol. The membrane and two sheets Whatman filter paper cut to the size of the gel were soaked in electrotransfer buffer. The membrane was positioned over the gel with care to avoid air bubbles between the gel and membrane. Bubbles were removed by rolling a clean pasteur pipette over the membrane. The membrane and gel were placed between pieces of filter paper and oriented with the gel toward the anode and the membrane toward the cathode in the Trans-blot Apparatus (Biorad). The transfer was run at 30 v, 0.10 amps for 18 h. Tap water was run via coiled tubing through the transfer chamber to maintain cool transfer temperature. After 18 h, the potential was raised to 45 v for 2-3 h.

The staining procedure described below applies to both electro-transferred protein and to blotted proteins (immunoblotting). The nitrocellulose or PVDF membranes bearing the antigens were rinsed briefly in PBS and were blocked for 30 m with 10 mM Tris-HCl, pH 7.4, containing 3 % BSA and 0.9% NaCl. After blocking, the membranes were washed with 3 changes of PBS. To the membranes was added mAb diluted 1:1000 (if ascites, diluted 1:100). The membrane was rocked for 1 h at 22^{o}C and washed 5 times with PBS, 5 m per wash. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Hyclone) was added 1:3000 and the membranes rocked for 1 h at 22^{o}C. The membranes were washed 4 times in PBS and substrate, 1,4-chloronapthol and H_2O_2, was added and the color allowed to develop. Substrate was made by combining immediately before use a

solution of 15 mg 1,4-chloro-napthol in 15 ml methanol with a solution of 70 ul 0.3% H_2O_2 in 25 ml PBS.

Enzyme-linked immunosorbant assay

Antigens were coated on polystyrene 96-well plates (Falcon) at a concentration of 2.5-10 ug/ml in 0.1 M sodium carbonate buffer, pH 9.6, and incubated 18 h at 22°C. The uncoated sites were blocked by incubating the wells with 1% human serum albumin (HSA) in phosphate buffered saline (PBS), pH 7.4 for 3 h at 22°C. Plates were washed 15 times with PBS containing 0.01% Tween 20. Ascites or mAb was serially diluted in PBS and incubated 100 ul/well for 1 h. After washing, horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Hyclone, Logan, UT) 1:10,000 (0.2 ug/ml) in PBS containing 0.5% HSA were added and incubated for 1 h. The plates were washed and the substrate containing 2,2 azinodi-(ethyl benzthiozoline sulfonic acid) (EBS) and H_2O_2 was added. The substrate was made fresh by combining 1:1 a solution of 2 ug/ml EBS in citrate buffer, pH 4.1 with a 0.3% H_2O_2 solution in water. After 10, 20, 30, and 60 m the plates were read at 405 nm in a Dynatech (Alexandria, VA) model 600 ELISA reader.

Competitive inhibition of ELISA

Competitive inhibition was performed by two different methods. In the first, 50 ul mAb in PBS was incubated (1 h at 37°C, sometimes followed by 18 h at 4°C) with 50 ul antigen in PBS resulting in final mAb concentration at 0.25 pmoles and final antigen con-

centration ranging from 0.005 to 2000 pmoles. The mAb was also incubated without inhibitor added to determine the effect of incubation on mAb activity. The second method was designed to enhance the inhibition of the mAb by low molecular weight antigens. In this case inhibition (using same concentrations and volumes as above) was performed in C1q-coated ELISA plates in order to selectively remove antigen-bound mAb. C1q-coated ELISA plates were prepared as described in "C1q purification". Antigens (SCM polypeptides purified by HPLC and streptococcal M 12 protein) were incubated with the mAb in C1q-coated wells for 1 h at 37°C. The individual well supernatants were transferred to SCM-coated plates to assay mAb activity in ELISA. The effect of C1q on unabsorbed mAb was measured by incubating the various dilutions of mAb in C1q-coated wells and transferring the supernatants to SCM-coated plates to measure mAb activity. Inhibition was also run under identical conditions in ELISA plates coated with BSA instead of C1q.

Comparison of native and denatured polypeptide antigens

The reactivity of native and denatured SCM peptides with mAb was compared by ELISA. ELISA plates were sensitized with fractions boiled for 30 m or with untreated fractions. ELISA was carried out as described.

Analysis of mAb/antigen interactions by equilibrium dialysis

The affinity and valence of the mAb for SCM antigens was determined by equilibrium dialysis.

The mAb/antigen interaction under study is that involving a multivalent antibody and antigens of unknown, possibly multiple valence. Thus, the following theoretical considerations apply to the analysis of the interaction between an antibody of valence n and antigen of valence s (Hardie and van Regenmortel, 1975). Let

A	= total antigen concentration, moles/liter
s	= antigen valence
sA	= total number of antigen binding sites, mole sites/liter
B	= total antibody concentration, moles/liter
n	= antibody valence
nB	= total number of antibody binding sites, mole sites/liter
y	= bound antibody concentration
x	= bound antigen concentration

At equilibrium the total number of occupied antibody sites (ny) is equal to the total number of bound antigen sites (sA).

That is, $ny = sx$ or $x = ny/s$

Thus the free antigen (mole sites/liter) is

$$sA - sx = sA - ny$$

and the free antibody (mole sites/liter) is

$$nB - ny = nB - sx$$

Under equilibrium conditions

$$(sA - sx) + (nB - sx) \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} sx \qquad (1)$$

By the law of mass action

$$K_a = k_{12}/k_{21} = \frac{sx}{(sA - sx)(nB - sy)} \qquad (2)$$

or

$$K_a = \frac{ny}{(sA - ny)(nB - ny)} \qquad (3)$$

Dividing (2) by B/B

$$K_a = \frac{sx/B}{(sA - sx)(n - sx/B)} = \frac{x/B}{(A - x)(n - sx/B)}$$

$$= \frac{r}{C(n-sr)} \quad \text{where } r = x/B \text{ and } C = A - x$$

$$r/c = K_a(n-sr) \qquad (4)$$

Equation (4) is the Scatchard form of the Law of Mass Action. From equation (4) the effect of antigen valence upon affinity can be seen. For the simplest case, antigen valence = 1, the equation reduces to $r/C = K_a(n - r)$. Plotting r/C vs. r over a range of antigen concentrations yields a straight line, giving the antibody valence n (intercept) and the K_a (slope). But at low antigen concentrations, this line often deviates from linearity and curves toward the x -axis. Thus, interpolation of the line to obtain the value for antibody valence is error prone. For this reason, least squares non-linear regression analysis was performed using the software program Enzfit to fit the binding data to equation 5. The free unbound antigen (C) was plotted against the bound antigen (r = x/B) and the antibody valence and K_d (= $1/K_a$) calculated.

$$r = \frac{nC}{K_d + C} \qquad (5)$$

For a multivalent antigen (s>1) equation 5 becomes

$$r = \frac{n/s\ (C)}{K_d/s + C} \qquad (6)$$

The least squares analysis yields n/s and K_d/s so that the antibody

valence and dissociation constant can not be determined without an independent estimation of the antigen valence, s.

Equilibrium dialysis procedure.

The time required to establish equilibrium for antigens alone was first determined. Several equilibrium dialysis cells were prepared containing different concentrations of SCM antigens in borate-buffered saline (BBS), pH 8.4. One side of the dialysis chamber containing 1 ml of antigen was separated by dialysis membrane (MW cutoff 10 kd) from the other chamber containing 1 ml BBS alone. The dialysis chambers had a volume greater than 1 ml and thus contained air bubbles to facilitate mixing. The chambers were maintained at 37^{o}C and equal volumes were removed from each side of the chambers at 0, 5, 10, 30, 60 m, then every 60 min for 8 h and at 18 h. Samples were frozen for determination of free antigen concentration as will be described in detail below. The time required for stable antigen concentration to be reached was used as the time for equilibrium in subsequent experiments.

The antigens then were dialyzed _vs_. mAb. Various concentrations of antigen were dialyzed against a constant concentration of mAb (2.5×10^{-6} M). Samples were removed from both sides at various time points and the free antigen concentrations determined at equilibrium.

To control for non-specific binding of the mAb, the antigens were dialyzed against TEPC (Sigma), an IgM myeloma. The equilibrium curve of antigens _vs_ buffer and _vs_ TEPC could be compared to the

curves obtained with antigens vs. mAb to show that the equilibrium of the antigens was specifically affected by the mAb.

To control for antigen specificity of the anti-SCM mAb, aprotinin (Sigma), MW 6,500, was dialyzed vs. the mAb and vs. buffer. The equilibrium curves were compared to determine the effect of the mAb on the equilibrium of a similar-sized but unrelated antigen. Aprotinin concentrations were determined from standard curves obtained by reading the absorption of various concentrations at 278 nm in a Gilford spectrophotometer.

Determination of free antigen concentrations for equilibrium dialysis

Free SCM antigen concentrations were determined by a sandwich ELISA employing solid-phase rabbit anti-SCM antibody as the capturing antibody and the anti-SCM mAb to detect captured antigens. Rabbit anti-SCM 1:100 in PBS was coated on 96-well ELISA plates (Falcon) 100 ul/well for 2 h at 22°C. The well supernatants were removed and saved for further coatings. Various dilutions of SCM antigens were added (10^{-5} to 10^{-8} M) to the wells and incubated 1 h 22°C to obtain a standard curve. The equilibrium dialysis samples were added to the wells in the same volume as the standards. After incubation, the plates were washed 10 times with PBS containing 0.03 % Tween-20. The mAb 1:5000 was added 100 ul/well and incubated 1 h at 22°C. The plates were washed and horseradish peroxidase-conjugated goat anti-mouse IgM (Hyclone) 1:10,000 in PBS was added 100 ul/well for 1 h at 22°C. The plates were washed and the sub-

strate was added and plates read as described in "Enzyme-linked immunosorbant assays". The free antigen concentrations in the equilibrium dialysis samples were determined by examining the optical densities obtained with the known concentrations of the antigens.

To determine that the solid-phase rabbit anti-SCM was not binding the anti-SCM mAb or the goat anti-mouse IgM, the plates with solid-phase rabbit anti-SCM were incubated with these antibodies but without antigen.

Amino acid analysis

Antigens were subjected to acid hydrolysis and amino acid analysis using the Beckman System 6300 High Performance Analyzer with the Na-buffer ion exchange column (Beckman). Samples were hydrolyzed in evacuated N_2-flushed test tubes containing 0.3 ml of 5.7 M constant boiling HCl, at 110°C for 4-6 h. Typically, 20 to 100 ug protein samples were hydrolyzed. After hydrolysis, tubes were opened and the contents dried over NaOH in a vacuum dessicator. Samples were solubilized in Na-S sample buffer (Beckman). Raw data was reported as nmoles amino acid/ml from which the minimal molecular weights and number of each amino acid residue was calculated. The nmoles of each amino acid recovered in an analysis was multiplied by the respective residue molecular weight to calculate the ng of the recovered amino acid. The residue molecular weight is equal to the molecular weight of the amino acid minus 18 (molecular weight of one molecule water). The g of each amino acid residue per 100 g protein was then calculated. A minimum molecular

weight (MMW) for the protein was then calculated based upon these values:

$$MMW = \frac{\text{amino acid residue molecular weight}}{\text{\% amino acid residue in protein}} \times 100$$

The moles of each amino acid residue per 100 g protein was then calculated. The mole ratio (MR) was calculated as follows:

$$MR = \frac{\text{moles of residue per 100 g protein}}{\text{moles of residue present in smallest amount}}$$

Corrected minimum molecular weights (CMMW) were then calculated based upon each amino acid:

$$CMMW = (MMW)(MR)$$

Monoclonal antibody

Analysis of ascites revealed that the mAb was IgM. Two approaches were undertaken to purify the mAb from ascites. The first was by affinity chromatography using two different columns (Reactigel and CNBr-Sepharose) containing SCM polypeptides.

Ascites was loaded on each column. Recorder tracings (recording absorption at 278 nm) showed that acid washes did not elute material from the Reactigel column while acid washes of the CNBr-Sepharose column eluted material in amounts directly related to the volume of ascites loaded (Figure 1). IgM was detected in the effluents of both columns. Though 88 ug/ml protein was detected in the eluent of the CNBr-Sepharose column, mAb activity was recovered only in the effluents of both columns.

Purification of mAb by affinity chromatography was not efficient and did not yield active mAb. For these reasons, euglobulin precipitation of IgM mAb from ascites was undertaken. Cellulose acetate electrophoresis of the starting ascites, the supernatant of the euglobulin precipitate, and the euglobulin precipitate showed that ascites ID8 contained the typical serum profile with albumin, α_1, α_2, β_1, β_2, and τ proteins with a strong immunoglobulin band due to high concentration of mAb. The supernatant showed all serum proteins except the immunoglobulin band. The precipitate showed a strong immunoglobulin band and no albumin or other detectable ascites proteins. The precipitate was subject to SDS-PAGE followed

by silver staining to assess the purity of the precipitate by a more sensitive technique. The reducing gel revealed one band at 65-70,000 M_r and one at 25-28,000 M_r, corresponding to the M_r of the heavy and light chains of IgM (Figure 2). Therefore this purification scheme produces purified IgM mAb essentially free of albumin and other mouse ascites proteins.

Anti-SCM activity was recovered only in the precipitates and was absent in the supernatants (Figure 3). Therefore the purified mAb retained anti-SCM activity.

The concentration of IgM was 2.21 mg/ml as determined using goat anti-mouse IgM antibody. This agreed with the BCA protein assay which showed the concentration of protein to be 2.10 mg/ml. These results further establish the purity of the IgM mAb.

Basement membrane

Microscopic examination of this sediment confirmed the presence of glomeruli free from cellular debris. This observation is consistent with the results obtained by Carlson et al. (1978) and Lange (1980a).

The efficiency of trypsin digestion of murine GBM was determined by measuring soluble protein in the supernatants of the reaction. Efficiency was of GBM digestion was 79-99% (Table I). Human lung and kidney BM also proved highly soluble with trypsin.

Figure 1. Attempt at purification of mAb on SCM-Sepharose column. CNBr-activated Sepharose was coupled with SCM. Different volumes of ascites were loaded on this column, and the bound material eluted with Glycine-HCl, pH 2.0.

AFFINITY PURIFICATION OF ID8 ON SCM COLUMN (SEPHAROSE)

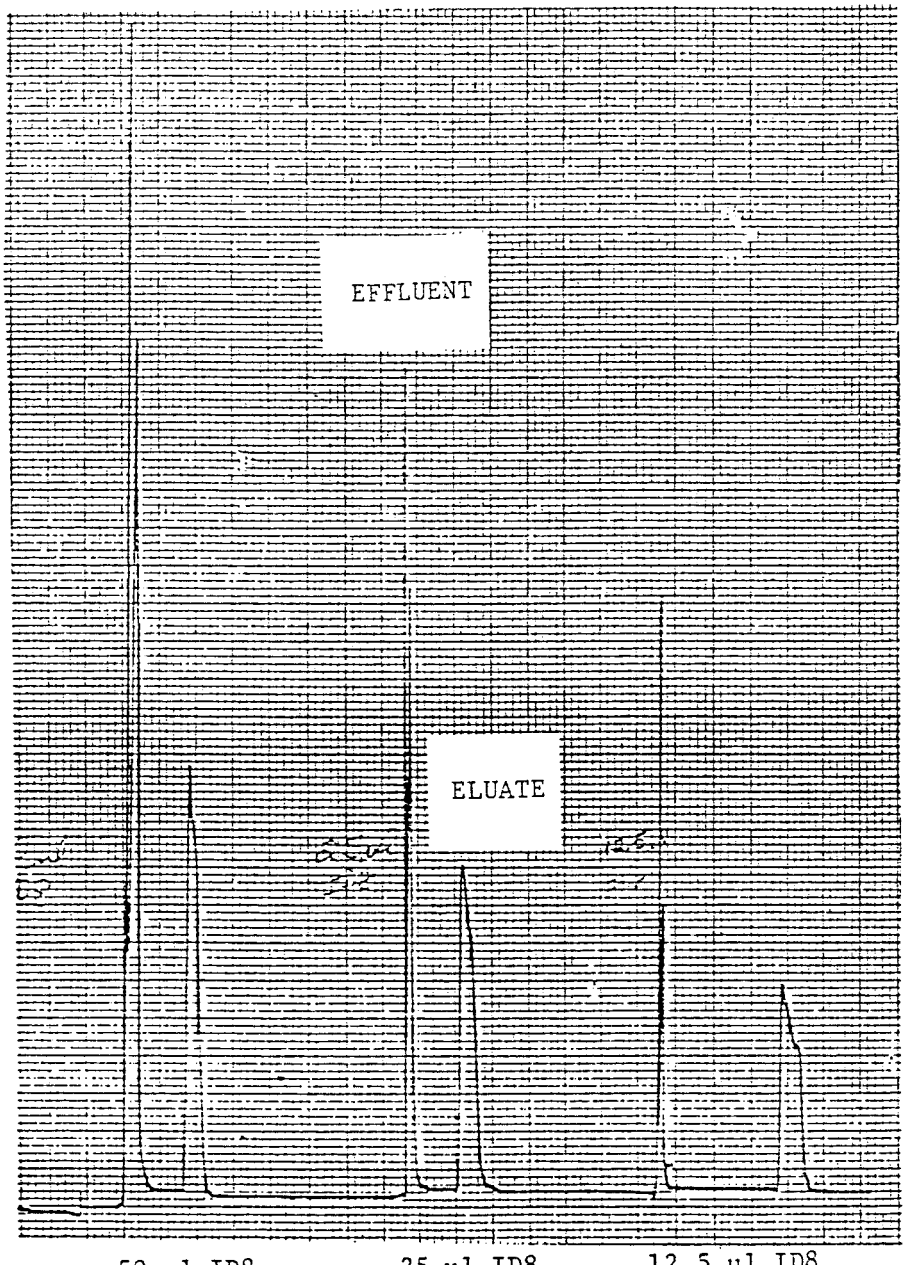


Figure 2. SDS-PAGE evaluation of purified IgM mAb. Lane 1: mol. wt standards. Lane 2: euglobulin precipitate.


A
B
97kd
66kd
42kd
31kd
21kd
14kd

Figure 3. Anti-SCM activity of the euglobulin precipitate. The IgM precipitate was resuspended in the original volume and its reactivity compared to that of the starting ascites and the euglobulin supernatant. Each bar represents the mean and standard deviation of three determinations.

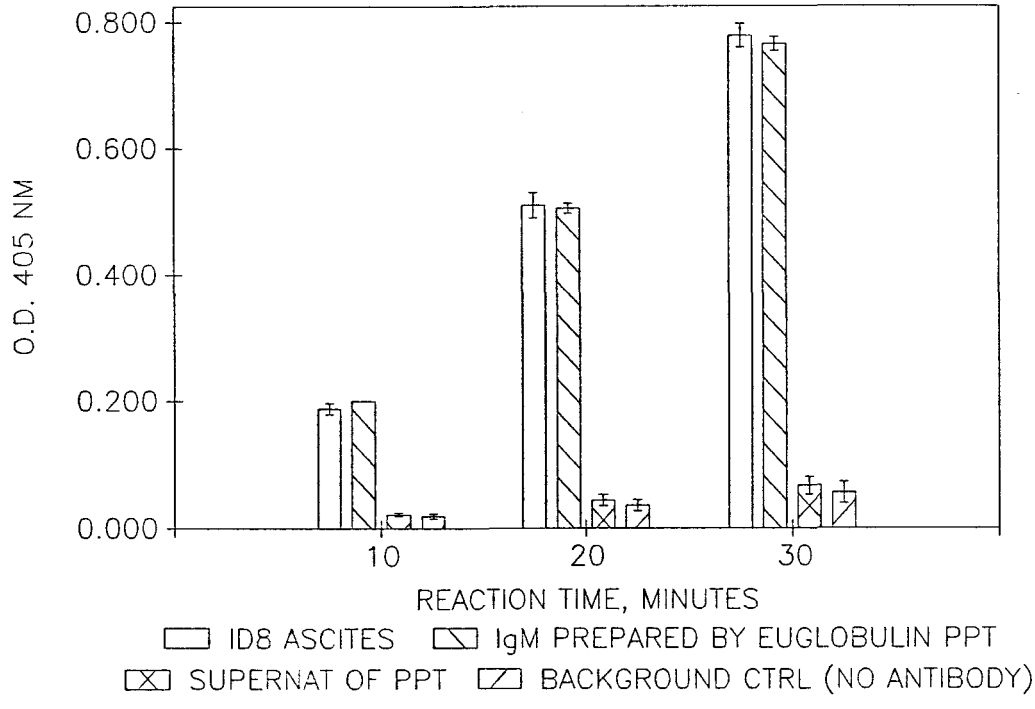

0.800
0.600
0.400
0.200
0.000
O.D. 405 NM
10
20
30
REACTION TIME, MINUTES
ID8 ASCITES
IgM PREPARED BY EUGLOBULIN PPT
SUPERNAT OF PPT
BACKGROUND CTRL (NO ANTIBODY)

TABLE I. YIELD OF SOLUBLE BM ANTIGEN

Tissue	Age	% BM Digested by trypsin
Murine kidney	Day 1	99
	Day 5	97
	Day 10	94
	Day 15	79
	Adult	89
Human kidney	Adult	80
Human lung	Adult	80

GBM or LBM was isolated from the tissues indicated and digested with trypsin. The per cent BM solubilized by trypsin is indicated.

Streptococcal cell membrane

Differential centrifugation was used to separate whole cells, debris and wall from membrane (Table II). Microscopic examination revealed that centrifugation at 3000xg pelleted most of the whole cells and some wall material. The supernatant from this was then centrifuged at 4500xg to remove the remaining wall material. The resulting supernatant was centrifuged at 24,500xg to pellet the membrane. Gram stain and wet mounts of this pellet showed no evidence of wall material. To confirm that the membrane pellet was wall-free, the membrane preparation was assessed for the presence of rhamnose and M 12 protein.

SCM prepared by this method did not inhibit anti-M 12 antisera from binding M 12-coated ELISA plates (Figure 4). The SCM preparation was 0.07% rhamnose, within the accepted limits for wall-free streptococcal membrane (Freimer, 1963).

SCM extracted with SDS or digested with trypsin yielded antigens reactive with purified anti-SCM mAb as determined by immunoblotting (Table III). Extraction with Triton-X 100 did not yield reactive antigens.

Isolation of membrane antigens by affinity chromatography

Previous work from this laboratory (Fitzsimons et al. 1987) showed that SCM and GBM shared common immunologic determinants. This was demonstrated when Fitzsimons et al. (1987) prepared mAb against SCM which was cross-reactive with GBM. One aim of the present work was to isolate SCM antigens using this mAb.

TABLE II. PREPARATION OF STREPTOCOCCAL CELL MEMBRANE

Pellet	Phase Microsc.	Gram Stain	Rhamnose, %
3000xg (whole cells)	WHOLE CELLS	POSITIVE	n.d.
4500xg (wall)	DEBRIS	POS. & NEG.	3.0
24,500xg (membrane)	DEBRIS	NEGATIVE	0.07

Figure 4. Assay for the presence of M 12 protein in SCM. SCM and M12 were incubated with rabbit anti-M12 antibody 1 hr 37°C followed by 18 hr 4°. The supernatants were transferred to M12-coated ELISA plates. Points represent the mean and standard deviation of three determinations.

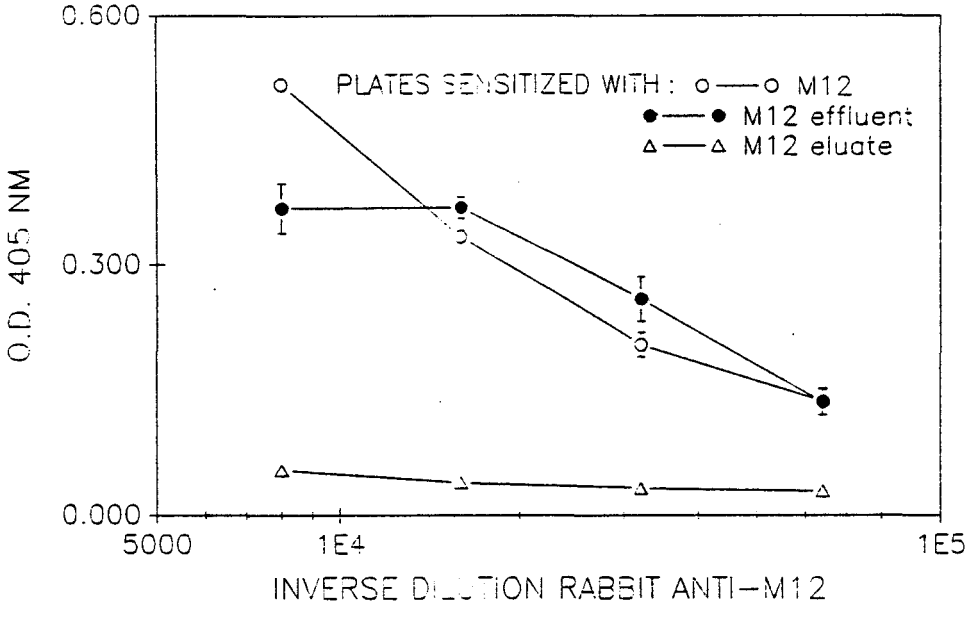

0.600
0.300
0.000
O.D. 405 NM
PLATES SENSITIZED WITH : o—o M12
●—● M12 effluent
△—△ M12 eluate
5000
1E4
1E5
INVERSE DILUTION RABBIT ANTI-M12

Figure 5. Determination of the rhamnose content of purified SCM. Rhamnose standards were used to determine SCM rhamnose content as described in Methods. The best fit was drawn through points representing single determinations.

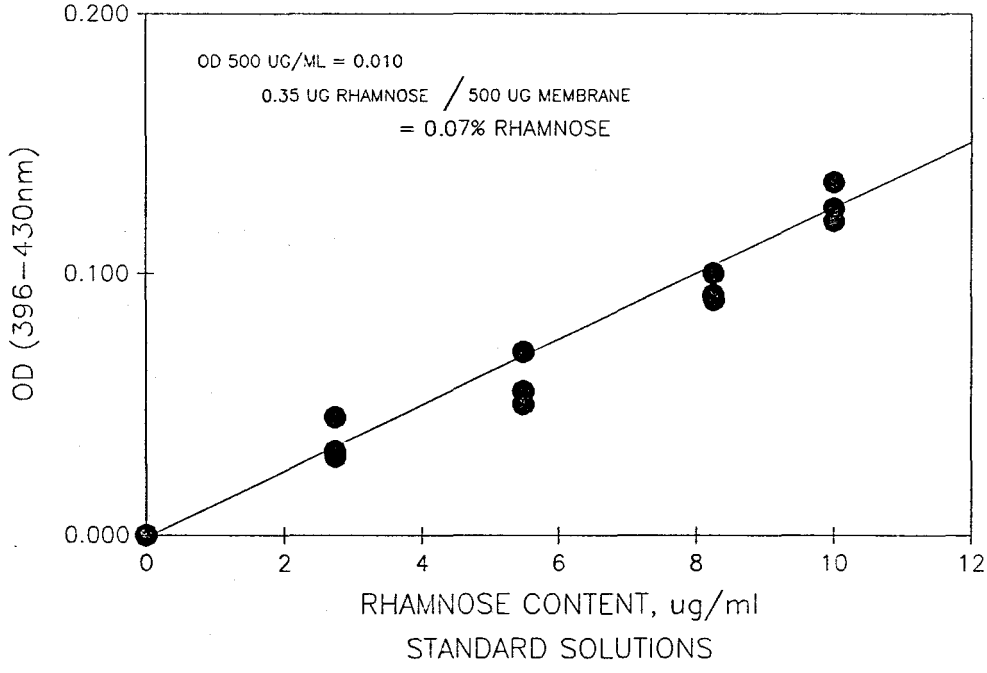

0.200
0.100
0.000
OD (396–430nm)
OD 500 UG/ML = 0.010
0.35 UG RHAMNOSE / 500 UG MEMBRANE
= 0.07% RHAMNOSE
0
2
4
6
8
10
12
RHAMNOSE CONTENT, ug/ml
STANDARD SOLUTIONS

TABLE III IMMUNOBLOT ANALYSIS OF SOLUBLE SCM ANTIGENS

mAb	Trypsin	EDTA	SDS	SDS/CNBr	Triton-X
ID8	3+	0	4+	2+	1+

Antigens extracted as indicated were blotted onto nitrocellulose at 10 mg/ml and probed with mAb 1:1000. Reactivity (substrate color development) is rated 0 to 4+.

Three mAb columns were prepared as described. Each column bound 31, 25, or 22 mg IgM (Table IV). These columns were assessed for specificity by loading various related and unrelated proteins. All BSA, MSA, M48 loaded could be accounted for in the effluent of the column (Table V). Further, amino acid analysis of the M48 eluate showed it to contain no protein, confirming that nonspecific binding to the mAb column did not occur (and suggesting that it was unlikely that mAb was being eluted by acid). Finally M12 protein was loaded on the column and the effluent and eluate assayed for the presence of M12 using rabbit anti-M12 antisera in ELISA. The reactivity of the effluent (concentrated to same volume as M12 sample loaded) was similar to the reactivity of M12 loaded; no M12 was detected in the eluate (Figure 6). These results confirm the specificity of the mAb and the affinity column.

The affinity column selectively bound from SDS-SCM and trypsinized SCM, GBM, LBM antigens of 20, 13, 11.2, 10 kd, respectively (Figure 7). From the molecular size and amount of the antigens bound from the tryptic digests and the amount of mAb coupled to the columns, the antigen/mAb molar combining ratios were calculated (Table VI). The antigen/mAb combining ratios varied for each antigen and were 2:1, 1:1, 3:1 for SCM, GBM, LBM, respectively. The antigen: antibody ratio was not determined for the SDS-extracted SCM antigen.

Because only one antigen was bound from each fairly complicated digest, trypsin-digested SCM was further fractionated by HPLC to determine whether the membrane contains additional antigens. In-

TABLE IV. COUPLING OF mAb TO TRESYL-ACTIVATED COLUMN FOR AFFINITY CHROMATOGRAPHY

	A.U. on	ml on	mg on	A.U. off	ml off	mg off	mg bound
COL 1.	1.30	25.00	25.00	0.031	25.00	0.60	24.40
	1.30	9.00	9.00	0.380	9.00	2.60	6.40
	0.38	9.00	2.60	0.380	9.00	2.60	0
						TOTAL BOUND	30.80
COL 2.	3.10	12.00	28.60	0.150	29.00	3.35	25.25
	0.15	14.50	1.67	0.150	14.50	1.67	0
						TOTAL BOUND	25.25
COL 3.	3.40	12.00	31.20	0.600	20.00	9.20	22.00
	0.60	10.00	4.60	0.600	10.00	4.60	0
						TOTAL BOUND	22.00

A.U. = absorbance units at 278 nm. IgM content determined from the absorption at 278 nm using extinction coefficient 1.3

TABLE V SPECIFICITY OF ANTI-SCM mAb AFFINITY COLUMN

Sample[a]		A.U.[b] On	A.U. EFFLUENT	A.U. ELUATE	% IN EFFLUENT
BSA	RUN 1.	0.400	0.405	0.000	100.00
	2.	0.320	0.300	0.000	100.00
M 48	1.	0.750	1.000	0.000	100.00
	2.	0.375	0.464	0.000	100.00
MSA	1.	0.130	0.138	0.000	100.00
	2.	0.250	0.245	0.000	100.00
CH_3COOH	1.	0.000	0.000	0.000	------
	2.	0.000	0.000	0.000	------

a Proteins, 1 mg/ml: BSA, bovine serum albumin; M48, Type 48 M protein; MSA, mouse serum albumin

b Absorbance units = Absorbance at 278 nm x ml

Figure 6. Specificity of anti-SCM mAb-coupled affinity column. M12 was loaded and the effluent and eluate collected. These were concentrated, resuspended in the volume originally loaded, and coated on ELISA plates. Rabbit anti-M12 antibody was used to detect the presence of M12. Points represent the mean and standard deviation of three determinations.

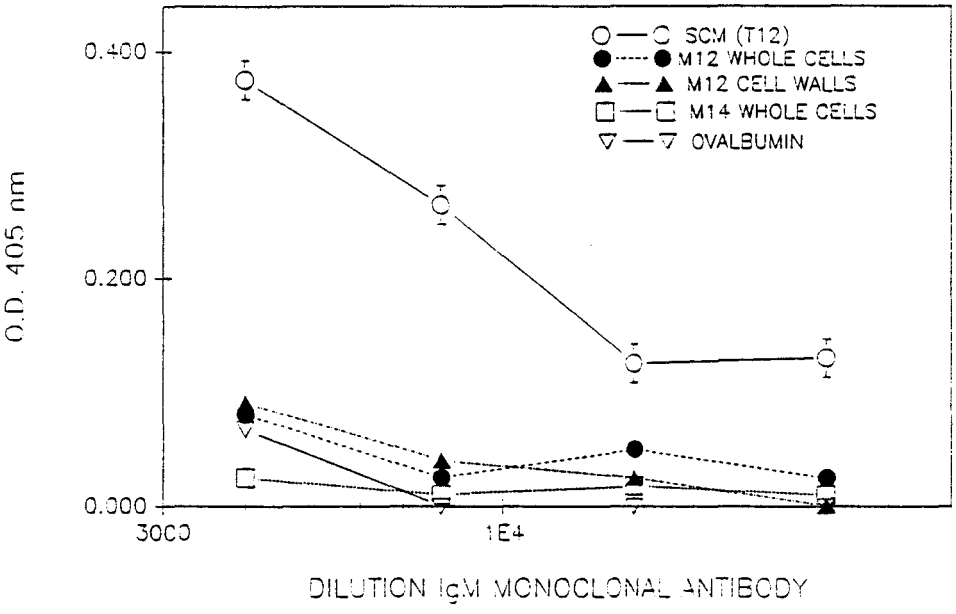
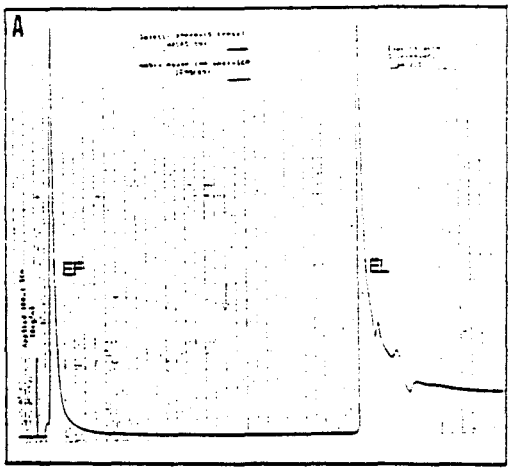
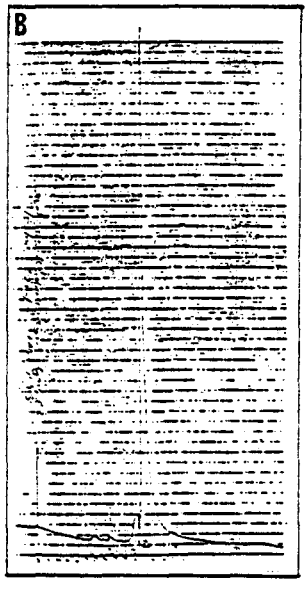
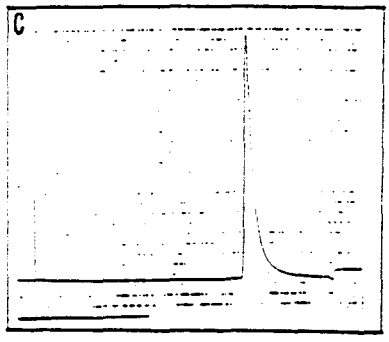
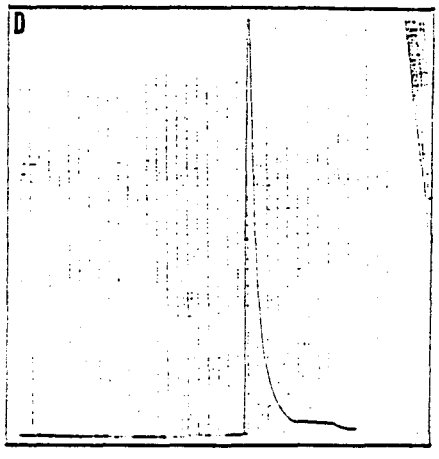

SCM (T12)
M12 WHOLE CELLS
M12 CELL WALLS
M14 WHOLE CELLS
OVALBUMIN
0.400
0.200
0.000
O.D. 405 nm
3000
1E4
DILUTION IgM MONOCLONAL ANTIBODY

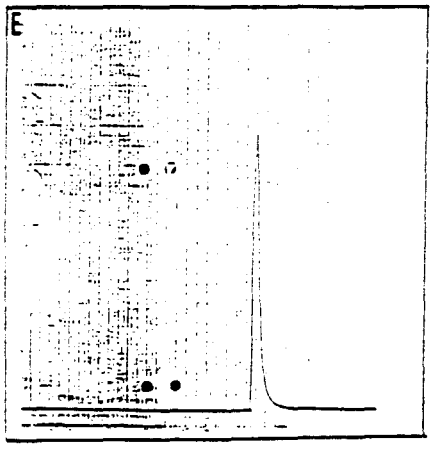
Figure 7. HPLC-affinity chromatography of membrane preparations. A: HPLC-affinity chromatographic preparation of antigens from trypsin-soluble SCM. B-E: HPLC-sizing of SLS-soluble SCM eluate (B), trypsin-soluble SCM (C), GBM eluate (D), LBM eluate (E).


A


B


C


D


E

Reactivity of SCM polypeptides with mAb in ELISA

To assess the reactivity of low MW SCM fractions which were not bound by the affinity column, ELISA microtiter plates were coated with 11.6, 9.2, 7.0, 4.7, 2.3 kd antigens at known concentrations. The plates bound approximately 90% of the protein at the coating concentrations employed (Table VII). First, the reactivity of each antigen was determined and shown to be proportional to the amount of antigen coated on the plate. Of interest, however, was the finding that equal ug amounts of these different size fractions gave similar reactivities with the mAb as judged by similar optical densities (Figure 10). Since the reactivity was proportional to antigen concentration and because equivalent reactivity occurred for equal amounts of different size antigens, it follows that these antigens differed in their molar reactivities. That is, reactivity of each antigen seemed to be directly related to antigen molecular size. Unfractionated trypsin-digested SCM, from which these polypeptides were derived, exhibited significantly higher reactivity in ELISA when present in the same ug amount as the individual polypeptides (Figure 10).

Important for interpretation of these results is the specificity of the mAb in the system. Specificity of the mAb for solid-phase antigen was tested on plates coated with a variety of antigens: lyophilized, heat-killed whole M-type 12 cells (from which SCM was derived), M-type 12 cell walls, M-type 14 whole cells, ovalbumin, and SCM from nontypable group A streptococci (Figure 11). The mAb showed significant reactivity in ELISA only with SCM anti-

gens; the mAb was relatively non-reactive with the related bacterial antigens and the unrelated protein ovalbumin (Figure 11). The mAb was also reactive with the nontypable SCM, indicating that the membranes from these strains share antigens, although these antigens appear to be at different concentrations or densities. This result also shows that the mAb does not recognize M protein determinants since mAb binds trypsin-digested membrane from the nontypable strain.

Affinity chromatographic analysis of SCM polypeptide antigens

These antigens were reactive with mAb in ELISA but were not detected in eluates of affinity chromatography (only a 13 kd antigen

Figure 8. HPLC profile of SCM tryptic digest. SCM tryptic digest was passed over the Zorbax gel filtration column and individual peaks sized and collected.

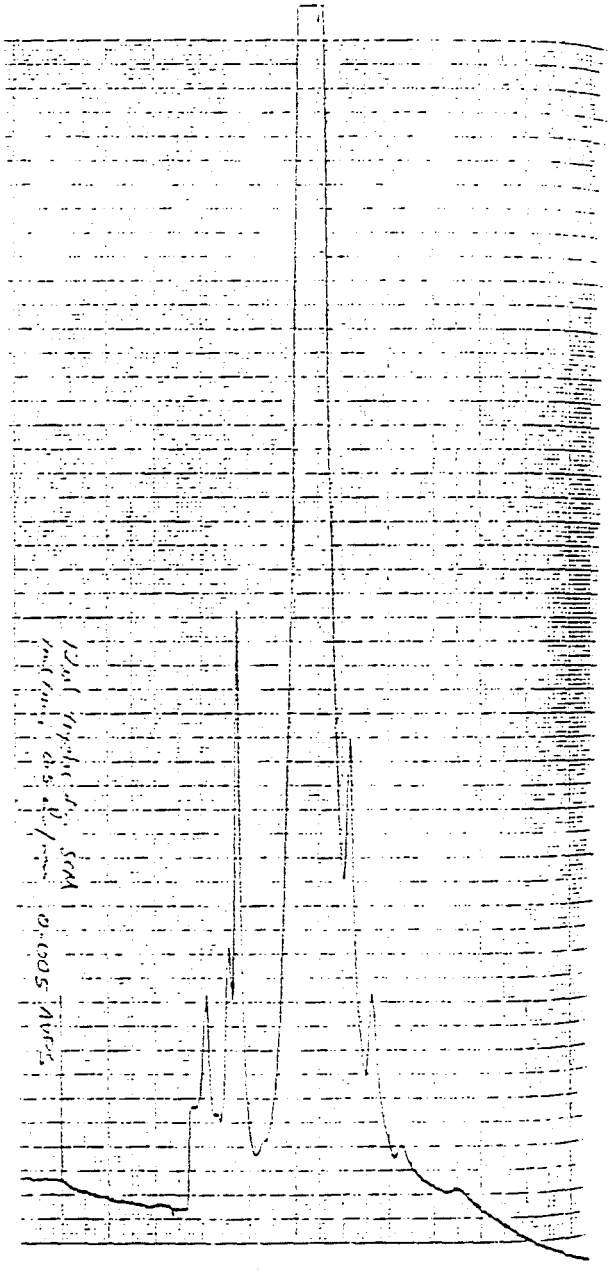


Figure 9. HPLC-purification of SCM polypeptide antigens. SCM tryptic digest was passed over GF-250 gel filtration column and fractions of mol. wt 11.6 (A), 9.2 (B), 7.0 (C), 4.7 (D), and 2.3 (E) purified by several rounds of recycling.

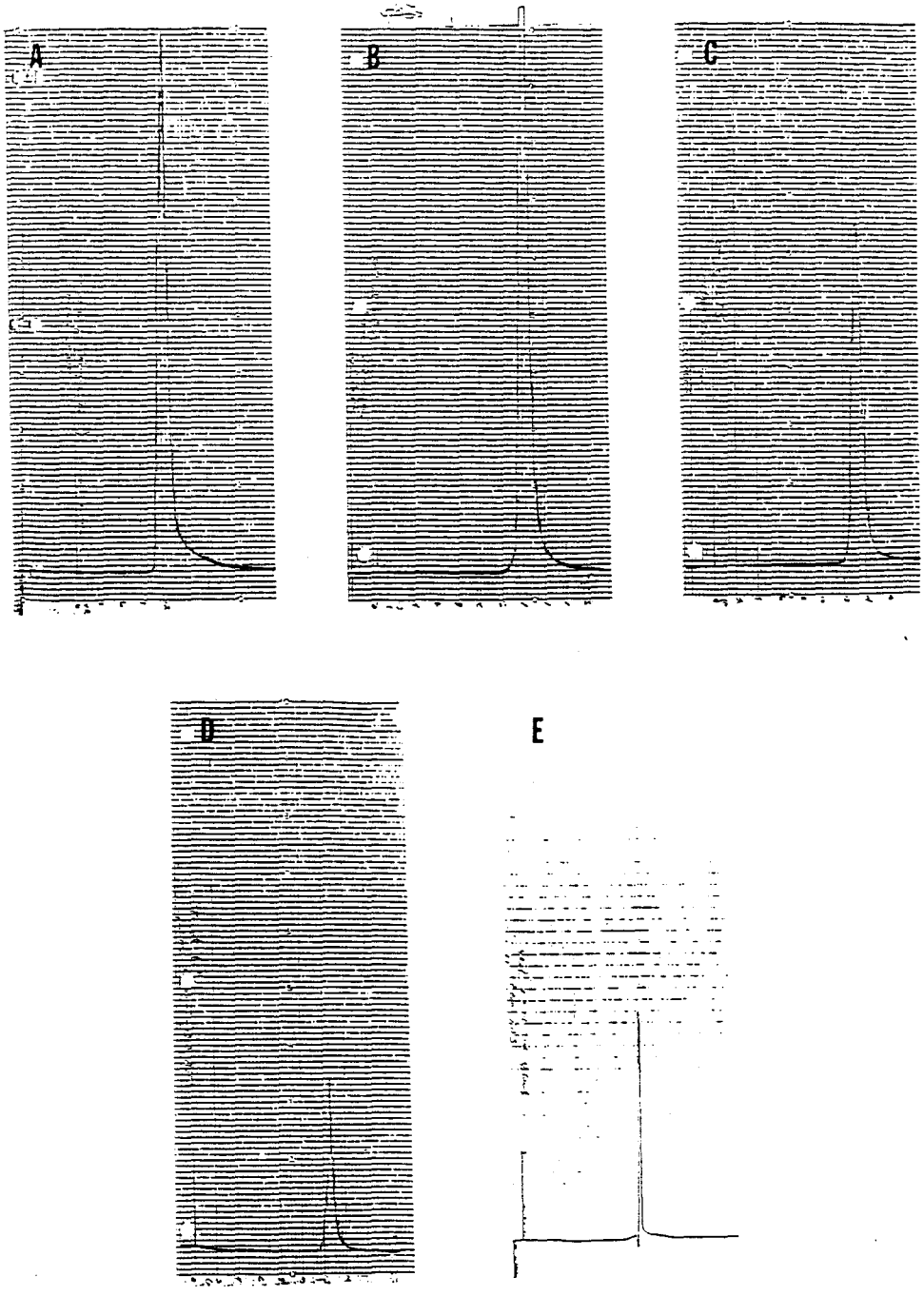

A
B
C
D
E

TABLE VII. ELISA MICROTITER PLATE-BINDING EFFICIENCY OF SCM POLYPEPTIDES

Protein, MW	Sensitizing Concentration, ug/ml	Per Cent Bound
9,200	20	>90
	10	>90
	5	85
7,000	20	>90
	10	>90
	5	75
4,700	20	>90
	10	>90
	5	81
2,300	20	>90
	10	>90
	5	75

Each antigen was coated on the plates at the indicated concentrations and the amount of protein remaining in the supernatant measured by BCA to calculate the per cent bound to the plate.

Figure 10. Reactivity of HPLC-purified SCM polypeptide antigens. Equal ug amounts of SCM and each size fraction were coated onto ELISA plates and their reactivities with the mAb (1:8000) compared. Each bar represents the mean and standard deviation of three determinations.

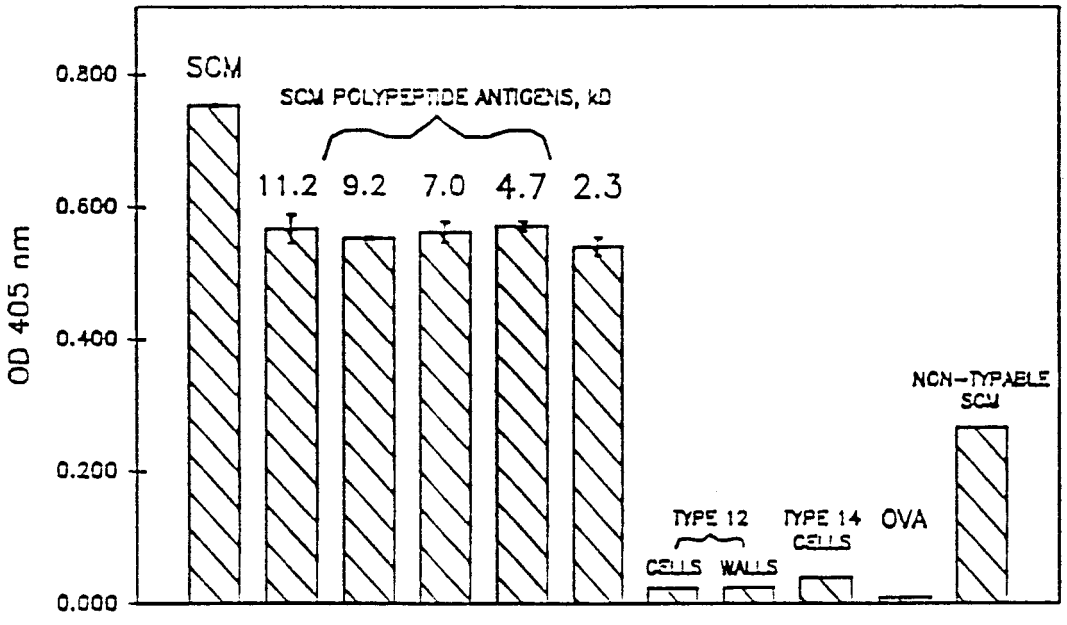
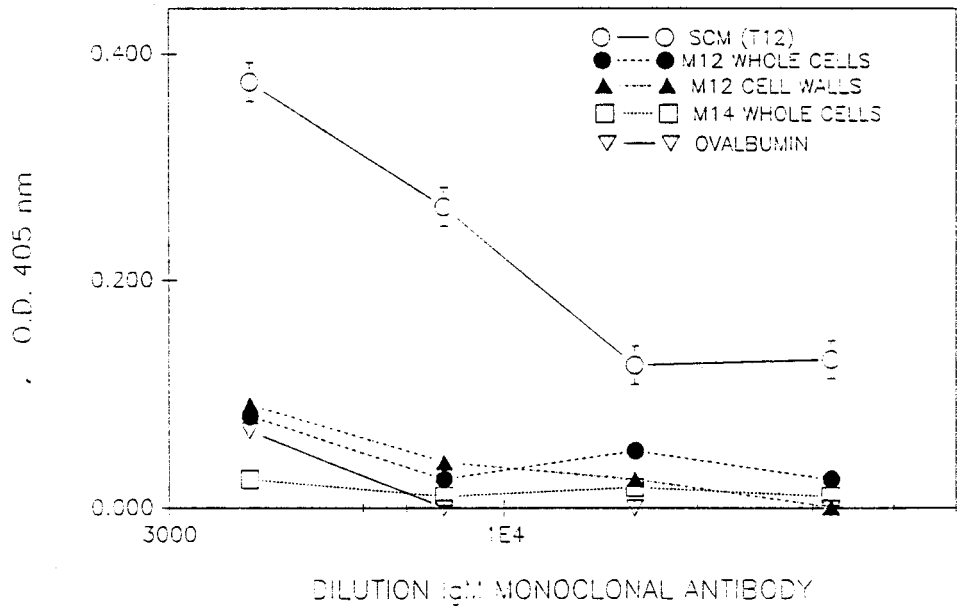

OD 405 nm
0.800
0.600
0.400
0.200
0.000
SCM
SCM POLYPEPTIDE ANTIGENS, kD
11.2
9.2
7.0
4.7
2.3
TYPE 12
CELLS
WALLS
TYPE 14
CELLS
OVA
NON-TYPABLE
SCM

Figure 11. SCM from group A nontypeable SCM was isolated as described for SCM. ELISA plates were coated with 5 ug/ml each control antigen indicated and assayed for reactivity with mAb.


SCM (T12)
M12 WHOLE CELLS
M12 CELL WALLS
M14 WHOLE CELLS
OVALBUMIN
0.400
0.200
0.000
O.D. 405 nm
3000
1E4
DILUTION IgM MONOCLONAL ANTIBODY

from trypsin digest and 20 kd from SDS-SCM were detected). However, each antigen did bind the affinity column when applied individually (Table VIII). The percent antigen in the eluate (the percent bound) for different amounts of antigen loaded was calculated; this number represents the efficiency of binding at different antigen concentrations loaded. The maximum efficiency for each antigen was achieved when approximately 40 ug of the antigen was loaded (Table VIII). Of interest, the maximum efficiency of antigen binding (43 - 90%) was directly related to antigen size (Table VIII). These binding characteristics are consistent with the size-related reactivities obtained by ELISA (Figure 10).

It is possible that the size-related binding efficiencies in affinity chromatography were due to size-dependent stability of conformational epitopes. Another possibility is that the binding characteristics simply reflected differences in the number of epitopes per unit antigen. The binding characteristics of these antigens in ELISA and in affinity chromatography are consistent with the interpretation that these antigens do vary in their number of epitopes available for mAb binding.

The use of solid phase antigens in ELISA introduces some variables which may affect the outcome of the experiments. Although the efficiency of antigen binding to the plates is high, the amount actually bound varies slightly and may affect the interpretation of the mAb binding results. In addition, the orientation of the antigen when bound to the plate may affect the accessibility of epitopes. Likewise, affinity chromatography utilizes immobilized mAb, which

TABLE VIII. BINDING OF SCM ANTIGENS TO mAb COLUMN

MOL. WT, kd	nM LOADED[a]	nM BOUND	% BOUND[b]
11.2	3.5	3.2	91
9.2	4.3	3.7	86
7.0	5.4	3.8	71
4.7	8.1	4.4	55
2.3	17.3	7.4	43

a Each peptide was HPLC-purified and 40 ug loaded. From the molecular weights the nanomoles loaded was calculated.

b The % bound was determined by measuring protein in the eluates with BCA and by comparing areas under the curves of the effluents and the eluates. Amount bound is the average of several experiments.

may affect optimal mAb/antigen interactions. In an attempt to overcome these potential problems, a liquid phase inhibition assay was designed in order to examine the antigen/antibody interactions.

Competitive inhibition of mAb by SCM antigens

The ability of the SCM antigens to absorb mAb was compared by competitive inhibition ELISA. Two different methods of competitive inhibition were used. In the first, designated the direct inhibition assay, different concentrations of SCM antigens were incubated with mAb for 1 h at 37°C followed by 18 h at 4°C. The mixture then was transferred to SCM-coated ELISA plates to assay mAb reactivity. In the second method, termed C1q-enhanced inhibition, the mAb was incubated with various concentrations of SCM antigens at 37°C in C1q-coated ELISA plates so that inhibitor-bound mAb would be selectively removed during incubation. Individual well supernatants were removed and tested for reactivity for SCM-coated plates.

Inhibition by the SCM antigens in the direct inhibition assay never exceeded 50% even at antigen concentrations in great excess (Figure 12A). If the mAb had a relatively low affinity for the liquid-phase inhibitor, mAb/Ag-inhibitor complexes may have easily dissociated due to higher affinity mAb binding to the solid-phase SCM antigens. To overcome this potential problem, the inhibition was performed in C1q-coated plates in order to selectively remove mAb/inhibitor complexes as they formed.

The binding of solid-phase C1q to free mAb (no antigen added) was measured and found to be negligible (Figure 13). Inhibition

performed in the presence of C1q was consistent and reached higher levels than when performed without C1q. Type 12 M protein, a streptococcal wall-derived antigen, did not inhibit the mAb (Figure 12B). The inhibition curve for each antigen shifted to higher antigen concentrations as the size of the SCM antigen decreased: the amount required for 50% inhibition of mAb was 0.30, 10, 40, and 110 pmoles for the 9.2, 7.0, 4.7, and 2.3 kd antigens, respectively (Figure 12B). These results are consistent with those obtained from direct ELISA (Figure 10) and affinity chromatography (Table VIII) which suggested that mAb binding was directly related to antigen size. Of note, relatively low concentrations of the 9.2 and 7.0 kd antigens achieved relatively high levels (20 - 40%) of inhibition, while similar concentrations of the 4.7 and 2.3 kd antigens had little effect on the mAb (Figure 12B). These high levels of inhibition by the larger antigens were not observed for non-C1q inhibition. C1q in this system may stabilize mAb/inhibitor interactions or bind with higher affinity IgM mAb complexed with multivalent antigens.

Inhibition was specific as described above using the M12 protein as a negative control. Cross-reactivity was confirmed by performing inhibition with low MW GBM polypeptide fraction (Figure 14). As little as 1 ng inhibitor produced over 90% inhibition of mAb activity.

Collectively, these results show that the SCM polypeptide antigens vary with molecular size in their reactivity with mAb: increasing antigen size was associated with increased mAb column binding; equal amounts of different size solid-phase antigens showed

Figure 12. Competitive inhibition of mAb by SCM antigens. A) SCM antigens were incubated with mAb 1 h 37°C as described in Methods. Supernatants were transferred to SCM-coated plates to assay mAb activity. The percent inhibition is the mean of three experiments. B) C1q-enhanced inhibition was performed by incubating the SCM antigens and mAb in C1q-coated plates. The supernatants were then transferred to SCM-coated plates to assay mAb activity. The results are the mean and standard deviation of three experiments.

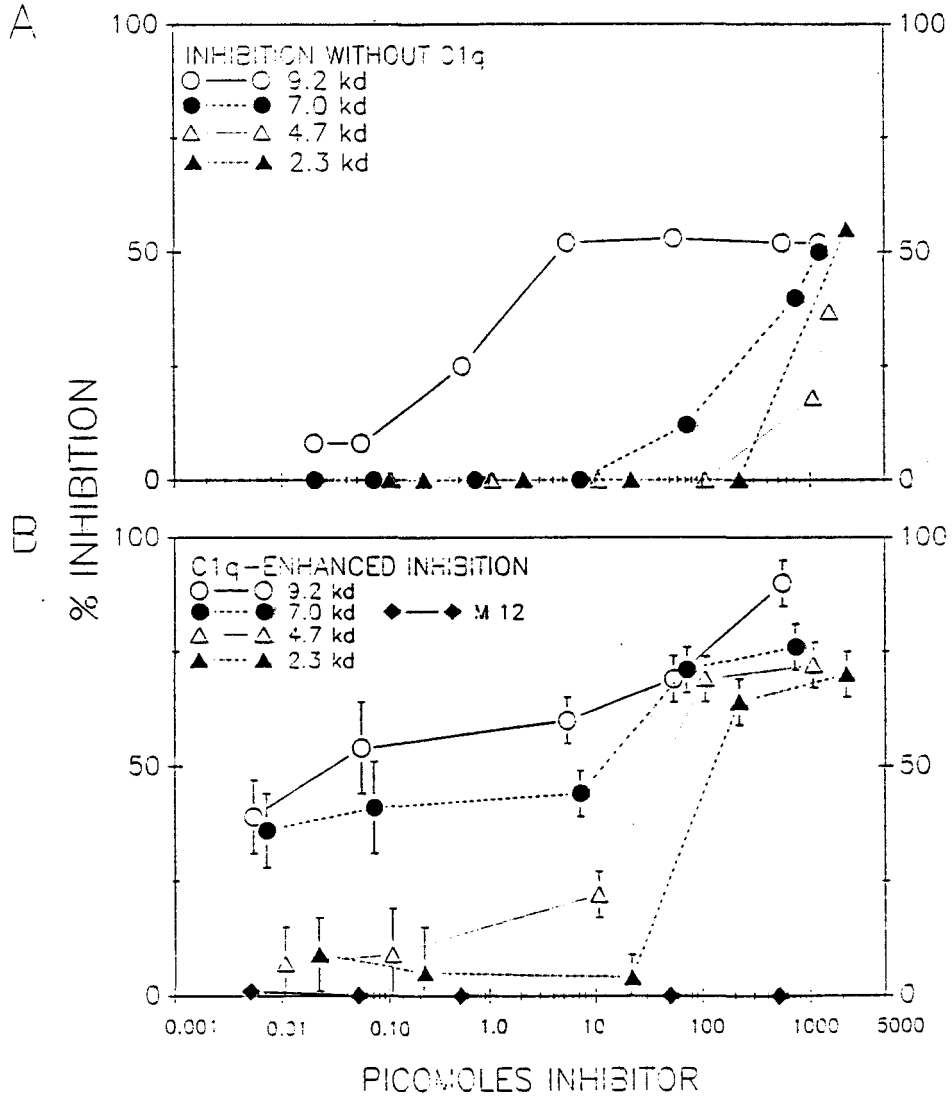

A
B
100
50
0
% INHIBITION
INHIBITION WITHOUT C1q
9.2 kd
7.0 kd
4.7 kd
2.3 kd
C1q-ENHANCED INHIBITION
9.2 kd
7.0 kd
4.7 kd
2.3 kd
M 12
0.001
0.01
0.10
1.0
10
100
1000
5000
PICOMOLES INHIBITOR

Figure 13. Measurement of free mAb binding to C1q-coated ELISA plates. mAb was incubated in C1q-coated wells and transferred to SCM-coated wells. Points represent the mean and standard deviation of three determinations.

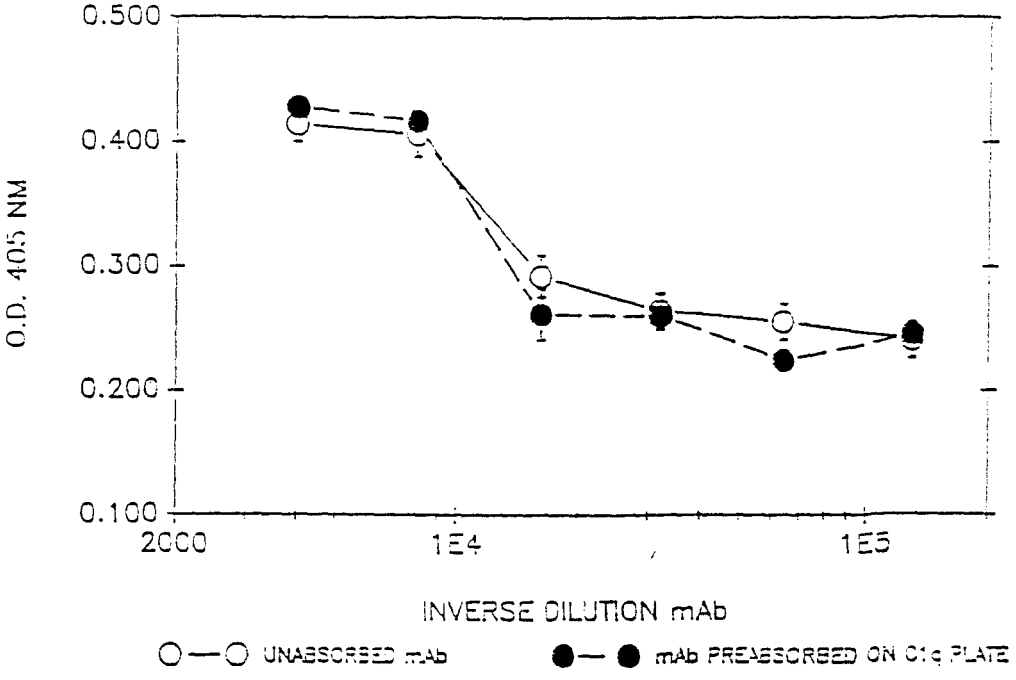
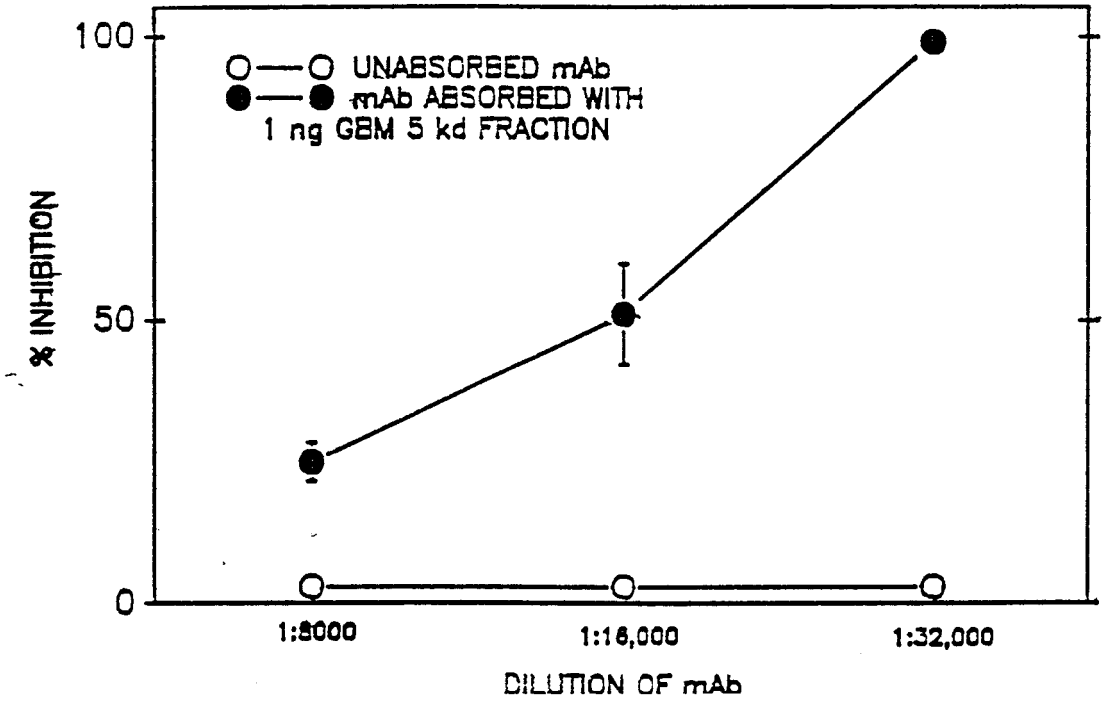

0.500
0.400
0.300
0.200
0.100
O.D. 405 NM
2000
1E4
1E5
INVERSE DILUTION mAb
UNABSORBED mAb
mAb PREABSORBED ON C1q PLATE

Figure 14. Competitive inhibition of mAb by low mol. wt GBM antigen. A 5 kd HPLC-purified GBM fraction was incubated with different dilutions of mAb 1 hr 37°C and 18 hr 4°. Supernatants were transferred to SCM-coated plates to assay mAb activity. Points represent the mean and standard deviation of three determinations.

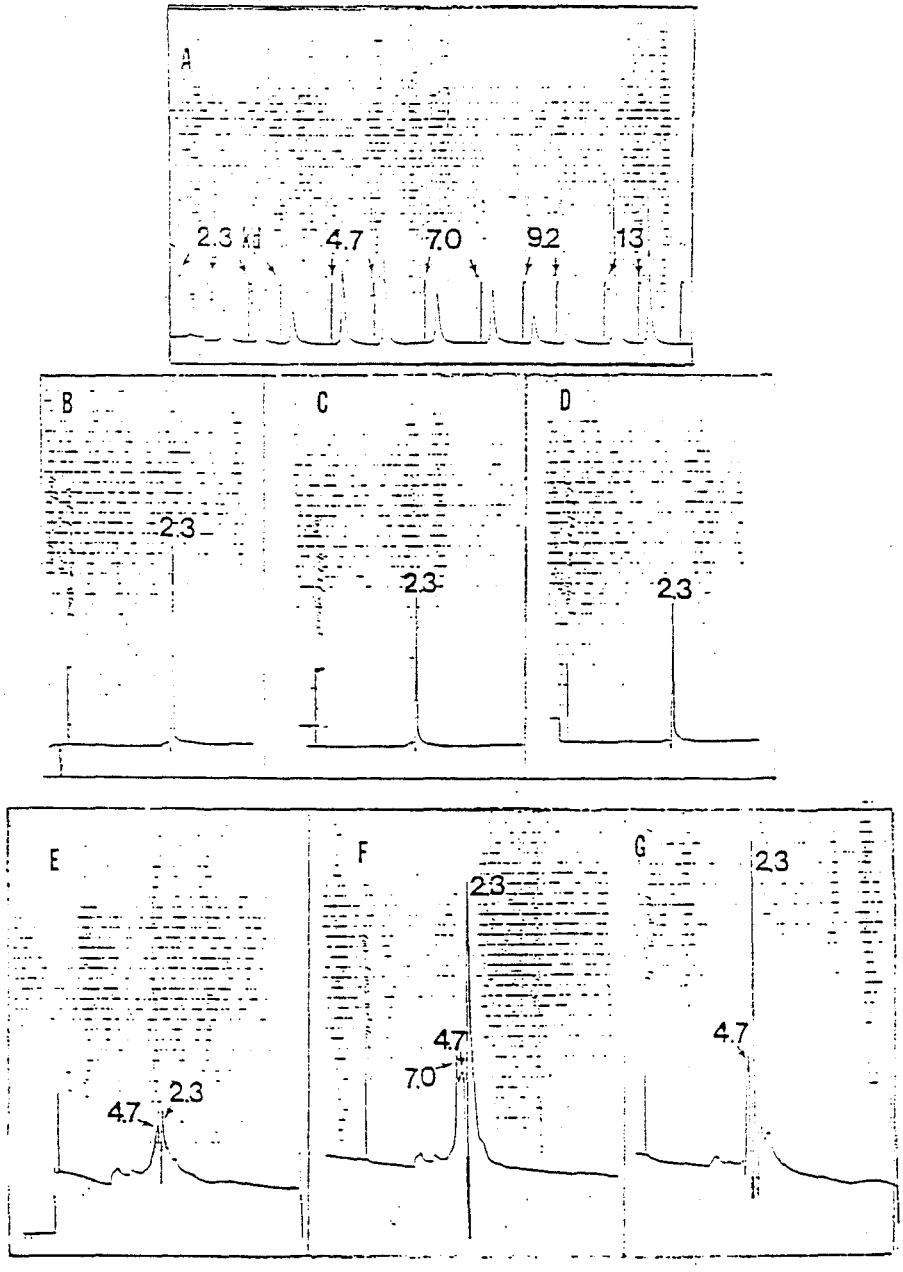

100
50
0
% INHIBITION
UNABSORBED mAb
mAb ABSORBED WITH
1 ng GBM 5 kd FRACTION
1:8000
1:16,000
1:32,000
DILUTION OF mAb

antigen size. Whatever the structural basis may be for the differences in reactivity with mAb, these differences might be expected to be manifested in the experimentally determined functional affinity of the mAb/antigen interaction.

Affinity of mAb for SCM polypeptide antigens

Several observations prompted an investigation into the affinity of interaction between the SCM antigens and the mAb. First, the antigens showed a size-dependent ability to inhibit the mAb; the amount required for inhibition increased with decreasing antigen size. Second, the mAb affinity column bound from the complete tryptic digest only a 13 kd antigen, yet the 11.2, 9.2, 7.0, 4.7, 2.3 kd SCM antigens each were reactive in ELISA. Finally, the SCM antigens each did bind the mAb column when applied alone, but the efficiency of binding decreased with decreasing antigen size. To determine whether these observations, particularly the differences in mAb column binding efficiencies, were due to differences in mAb affinity for these antigens, two different procedures were employed to measure mAb affinity. First, the ability of these antigens to compete for binding to the mAb column was measured. In this experiment, the ability of each size antigen to elute or displace other size antigens was determined. Second, a more direct measure of mAb affinity was obtained by equilibrium dialysis of mAb vs. each antigen.

Figure 15. Competitive binding of SCM antigens to mAb column. Each antigen was loaded with repeated injections as indicated in (A) until column was saturated. The effluent from each was analyzed by HPLC gel filtration. The effluent of the 2.3 (B), 4.7 (C), 7.0 (D), 9.2 (E), 13.0 kd (F), and acid wash (G) are shown and the components identified.


A
2.3
4.7
7.0
9.2
13
B
2.3
C
2.3
D
2.3
E
4.7
2.3
F
2.3
4.7
7.0
G
2.3
4.7

Competition of SCM antigens in affinity chromatography

If the results of affinity chromatography (only 13 kd bound from tryptic digest) were due to high mAb affinity for the larger antigens, then the low MW antigens may have been displaced from mAb by the largest antigen. To address this problem, the ability of each antigen to displace smaller antigens from the affinity column was measured. First, the 2.3 kd antigen was loaded on the column and recycled until the column was saturated. The effluent was collected and the 4.7 kd antigen was added to elute the 2.3 kd antigen. The flowthrough was collected and the 4.7 kd added in an attempt to saturate the column. This was repeated with the 7.0, 9.2, 13 kd antigens, after which the column was washed with acid. The effluents and the acid wash each were analyzed by HPLC gel filtration to determine the size of the components eluted (Figure 15).

When the column was saturated with the 2.3 kd, the effluents contained the 2.3 kd, as expected. Attempts to saturate with the 4.7 kd resulted in only some 2.3 kd appearing in the effluent. Following with the 7.0 kd, again saturation could not be achieved, as only 2.3 kd was detected in the effluents. Loading the 9.2 kd eluted 2.3 and 4.7 kd, but the effluents never contained 9.2 kd. Finally, the 13 kd was loaded. Its effluents contained only 2.3, 4.7, 7.0 kd (Figure 15).

It is difficult to interpret the results of this series of experiments. Evidence suggests that the column did not reach saturation with any (except 2.3 kd) of the antigens. Following saturation of the column with 2.3 kd, the behavior of the other

antigens was unpredictable and inconsistent. Apparently, once saturated, the column did not readily bind the larger antigens. Addition of the larger antigens resulted not in total displacement of the smaller, but in only incremental and incomplete displacement. Thus, if the previously bound antigens were never completely eluted by larger antigens, the result would be what was observed: inability to saturate the column with the larger antigens. Therefore, the displacements that occurred can not be used to quantitate affinities for the mAb.

Equilibrium dialysis of mAb and SCM antigens

Equilibrium dialysis of SCM antigens vs. buffer, mAb, and TEPC was performed to establish equilibrium conditions for the antigens alone and to show that the mAb had a specific effect on the equilibrium of the antigens. Figure 16 shows that equilibrium was reached by 3 h at 37°C in all cases. At equilibrium the antigen concentration on its side of the chamber was lowered only when dialyzed vs. mAb. Varying antigen concentrations then were dialyzed vs. mAb (2.5×10^{-6} M) and the free and bound antigen concentrations at equilibrium determined. Free antigen concentration was plotted vs. bound antigen concentration (Figure 17). Non-linear least squares regression analysis of the binding data calculated the mAb capacity and K_d for each mAb/Ag interaction.

It appears that mAb was saturated only when dialyzed vs. 9.2 kd antigen (Table IX). The mAb reached only 40% saturation with the 7.0 kd antigen and much lower with the 4.7 and 2.3 kd antigens.

Figure 16. Equilibrium dialysis of SCM antigens. SCM antigens were dialyzed *vs.* buffer, mAb, or TEPC at 37°C. Antigen concentrations were determined on rabbit anti-SCM plates as described. Points represent the mean of at least three determinations.

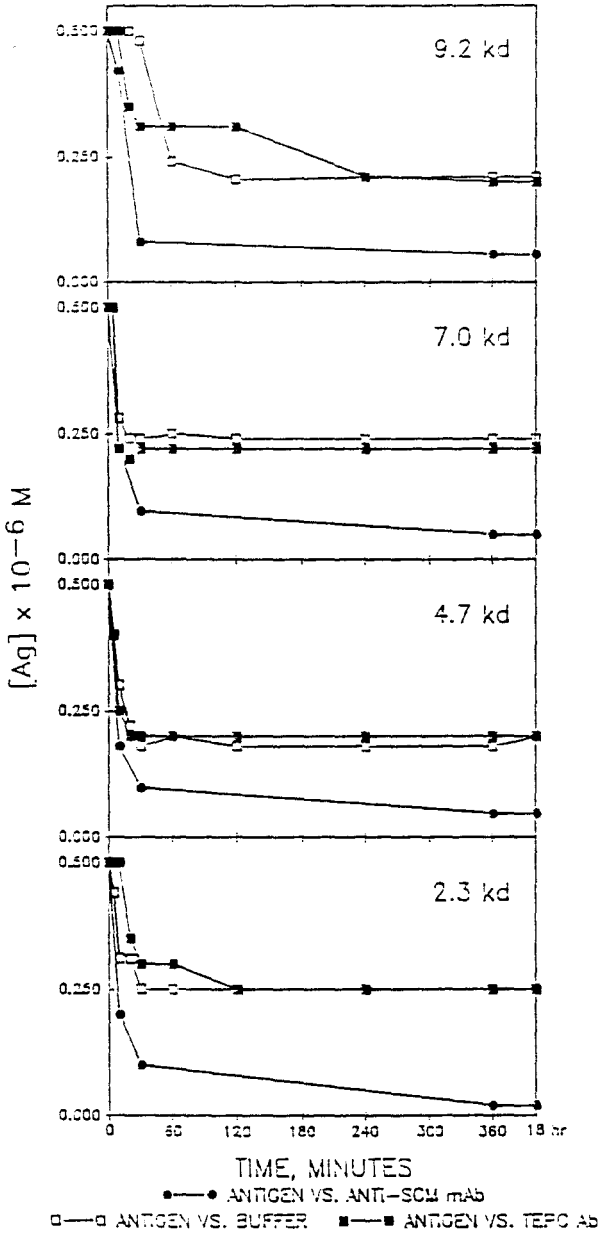
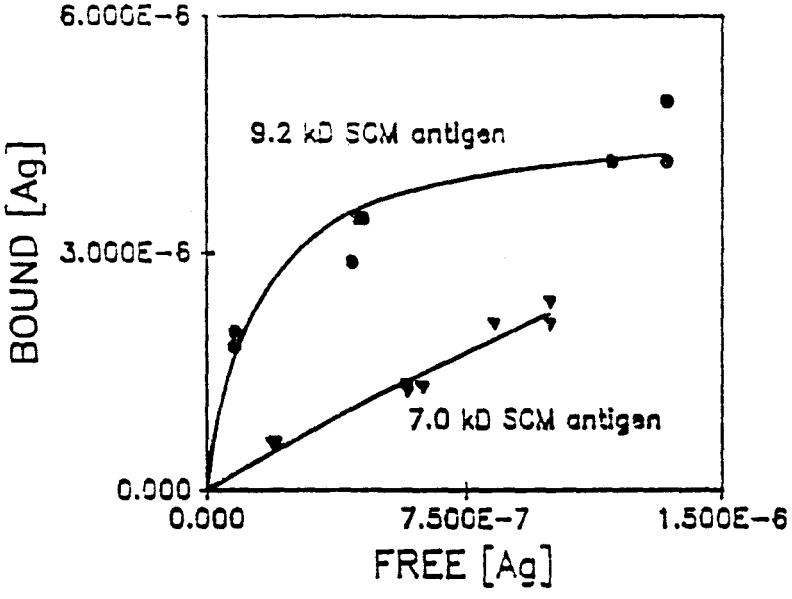

9.2 kd
7.0 kd
4.7 kd
2.3 kd
0.500
0.250
0.000
[Ag] x 10−6 M
0
60
120
180
240
300
360
18 hr
TIME, MINUTES
ANTIGEN VS. ANTI−SCIM mAb
ANTIGEN VS. BUFFER
ANTIGEN VS. TEPC Ab

Figure 17. Nonlinear least squares regression plots of binding data obtained by equilibrium dialysis. Antigen at different concentrations was dialyzed against a constant amount of mAb. At equilibrium the free and bound antigen concentrations were determined as described in Methods.

A


6.000E-6
3.000E-6
0.000
BOUND [Ag]
9.2 kD SCM antigen
7.0 kD SCM antigen
0.000
7.500E-7
1.500E-6
FREE [Ag]

B

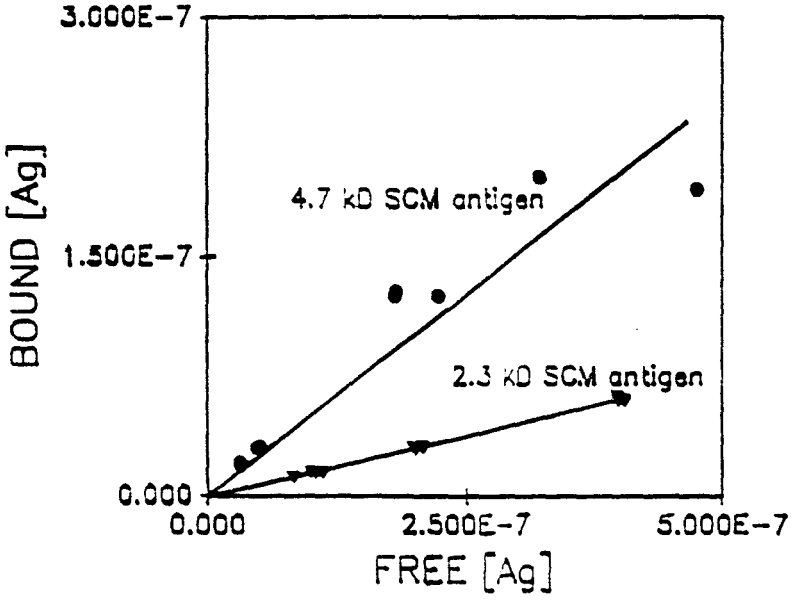

3.000E-7
1.500E-7
0.000
BOUND [Ag]
4.7 kD SCM antigen
2.3 kD SCM antigen
0.000
2.500E-7
5.000E-7
FREE [Ag]

TABLE IX. MONOCLONAL ANTIBODY AFFINITY AND VALENCE FOR SCM ANTIGENS

Ag, kD	mAb CAPACITY[a] (Moles)	mAb VALENCE[a]	SLOPE[a]	K_d ml^{-1}	%SATURATION OF mAb
9.2	4.69×10^{-6}	2 ± 0.1	0.029	1.39×10^{-7}	90
7.0	1.59×10^{-5}	6 ± 5	0.383	6.08×10^{-6}	40
4.7	n.d.	10[b]	0.462	3.83×10^{-6}[c]	8
2.3	n.d.	10[b]	0.153	1.15×10^{-5}[c]	1.2

a. Slope and capacity were calculated from plots of the binding data. Valence is defined as the capacity (max [Ag] bound divided by [mAb]).

b. Since the slope remains constant for the 4.7 and 2.3 kD antigens, but the per cent saturation decreases, K_d must increase and thus the capacity (valence) must also increase, likely approaching 10. See text.

c. K_d can not be determined as it was not possible to saturate the available antigen combining sites at the antigen concentrations available. Thus the values of K_d presented for the 4.7 kd and 2.3 kd antigens were calculated assuming a mAb valence of 10.

This degree of saturation places limitations on the interpretation of mAb valence and K_d. Because the mAb was saturated only in the case of the 9.2 kd antigen, mAb valence could be confidently measured only for this antigen. The valence of 2 agreed with the Ag:mAb combining ratio on the affinity column for the 13 kd antigen (Table VI). Although a direct measurement of valence cannot be derived with confidence for the two smallest antigens, it can be seen that the valence must increase for these smaller antigens. The slope remains constant for the 7.0, 4.7, and 2.3 kd antigens. Slope is defined as K_d/mAb capacity; since the slope is constant but K_d increases, the mAb capacity (valence) must also increase.

The affinity ($1/K_d$) of the mAb for these antigens was directly related to antigen size (Table IX). In the case of the 2.3 kd antigen, it is not clear whether an accurate K_d can be derived from the binding data since the mAb was so far from saturation. These results show an increase in affinity of the mAb for the larger antigens and suggests that the mAb valence may also be directly related to antigen size and observed reactivity.

Comparison of native and denatured SCM antigens

The antigen reactivity and size were found to be related to affinity of mAb/antigen binding. These observations could be explained by differences among the antigens in the expression, accessibility, or number of epitopes. The differences in mAb binding could be the result of a size-dependent stability of antigenic conformations. Instead, the size-related differences in mAb binding

TABLE X. REACTIVITY OF NATIVE AND DENATURED SCM ANTIGENS WITH mAb in ELISA

Antigen	Optical density, 405 nm ± S.D.		O.D. Increase
	Native	Denatured	
13 kd	0.236 ± 0.026	0.270 ± 0.027	0.034
11.2 kd	0.092 ± 0.020	0.348 ± 0.042	0.256
9.2 kd	0.103 ± 0.050	0.320 ± 0.050	0.217
7.0 kd	0.109 ± 0.027	0.326 ± 0.020	0.217
4.7 kd	0.108 ± 0.015	0.307 ± 0.023	0.199
2.3 kd	0.103 ± 0.005	0.231 ± 0.037	0.128

"Denatured" antigens were heated to boiling and coated on ELISA plates. The reactivity of these antigens with mAb was compared to untreated "native" antigens.

could be due to a repeating epitope in the amino acid sequence of these antigens. To distinguish between these two possibilities, that is, to test whether the epitope is defined by conformation or primary structure, SCM polypeptides derived from trypsin digest of SCM and the SCM antigen eluted from the affinity column each were subjected to heat denaturing conditions. Following boiling for 30 min each antigen retained reactivity with mAb. The untreated and denatured 20 kd antigen showed nearly the same level of reactivity (Table X). In contrast, the smaller SCM polypeptides achieved higher levels of reactivity after denaturing conditions (Table X). Of note, these antigens showed different responses to denaturing conditions: at antigen excess (antigen 5 ug/ml, mAb 0.25 ug/ml), the increase in reactivity was directly related to antigen size (Table X).

The epitope may be defined by amino acid sequence since heat denaturing conditions did not destroy the reactivity of these antigens. In fact, these antigens became more reactive following heat denaturation, suggesting that additional sequence-determined epitopes may become accessible after inter- and intra-peptide associations are disrupted.

Amino acid analysis

Amino acid analysis of these antigens was performed to determine if amino acid compositions could be correlated with the size-dependent reactivities. The amino acid analysis of each SCM antigen showed that the MW of each polypeptide as determined by amino acid

TABLE XI. AMINO ACID ANALYSIS OF SCM ANTIGENS

MOL WT		NUMBER OF EACH AMINO ACID																	
HPLC	AAA	B	T	S	Z	P	G	A	C	V	M	I	L	Y	F	H	K	R	ACID:BASIC
20 kd	18.9 kd	19	9	9	20	6	17	14	2	10	1	8	14	1	9	8	11	5	2.6
13 kD	11.9 kD	13	6	5	13	4	11	9	1	6	1	5	9	2	4	4	7	3	2
9200	9125	13	5	4	15	3	8	7	1	6	1	5	6	1	3	1	2	1	7
7000	6998	11	4	3	9	2	7	6	1	5	1	4	5	1	2	1	2	1	5
4700	4656	4	2	2	4	2	4	3	1	3	1	3	4	1	3	1	2	1	2
2300	2334	2	1	1	1	1	2	1	1	1	0	1	1	0	3	1	1	1	1

Acid:basic values were determined by dividing the total number of Glu and Asp by the total number of Arg, Lys and His. Single letter amino acid symbols: B Asp or Asn, T Thr, S Ser, Z Glu or Gln, P Pro, G Gly, A Ala, C Cys, V Val, M Met, I Ile, L Leu, Y Tyr, F Phe, H His, K Lys, R Arg

analysis correlated well with the MW obtained by HPLC gel filtration (Table XI). The nmoles/ml amino acid was reported for each antigen, from which the number of each amino acid per antigen was calculated (Table XI). From this analysis, it was clear that certain residues remained constant while other residues changed with changes in antigen size. All antigens contained one residue of Cys, Met, Tyr, His, and Arg except for the 2.3 kd antigen, which had no Met or Tyr. Each antigen had 3 Phe, except the 7.0 kd which had 2; each had 2 Lys, while the 2.3 kd had only 1. In contrast, the residues Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile and Leu each increased with increase in size of the antigens. Notably, acidic amino acids increased in number the most with increase in antigen size, resulting in differences in relative acidity of these antigens (Table XI) which can be seen when the ratio of acidic to basic amino acids is calculated for each antigen.

In these antigens, the number of acidic and aliphatic side chain amino acids was directly related to antigen size, reactivity and mAb affinity. Because evidence suggested that the epitope may be defined by amino acid sequence, one interpretation of the amino acid analyses is that these amino acids contribute to a linear epitope which repeats and thus is found in higher number in the larger antigens.

Amino acid sequencing

Several antigens were prepared by RP-HPLC or by SDS-PAGE and transfer to PVDF for amino acid sequencing. These results are sum-

marized in Table XII. Antigens initially isolated by HPLC gel filtration (2.3 and 4.7 kd SCM polypeptide antigens) did not absorb to C8 or C18 reverse-phase columns; nearly all the protein loaded appeared in the flowthrough with the phosphate salts. Different gradients did not change the absorption characteristics. The flowthroughs from the above runs were desalted on G10 columns; this material was sequenced and found to consist of low MW materials suggesting that the antigens may have been degraded by attempts at purification.

The 13 kd SCM antigen isolated by affinity chromatography and purified by gel filtration HPLC gave a single broad peak on the C8 column. Recycling on C18 showed a variety of low MW peptides. One peak containing approximately 30 pmoles protein was sequenced. The sample was found to be degraded to several low MW peptides by the purification step; peptide in the sample appeared to be N-terminally blocked. To reduce the handling steps of this antigen, affinity isolated 13 kd SCM antigen was placed directly on C18, C8, or C4 columns. These resulted in single broad peaks, which sequencing determined also to be contaminated by low MW peptides and di- and tripeptides.

Because the RP-HPLC buffers (acetonitrile and TFA) were suspected to be causing sample destruction, samples were prepared by SDS-PAGE and electrotransfer to PVDF. A 20 kd SCM antigen and a 13-15 kd SCM antigen were successfully transferred to PVDF in sufficient amounts for microsequencing. However, their sequences were not obtained due to N-terminal blocking of these polypeptides.

Finally, the 11 kd GBM antigen isolated by affinity chromatography was applied to the C18 column; the resulting single broad peak was recycled and sequenced. This sample also appeared to have been destroyed and/or N-terminally blocked.

As an internal control, β-lactoglobulin was prepared by RP-HPLC or by SDS-PAGE and transfer to PVDF. Sequencing of both preparations gave appropriate amino acid sequences.

TABLE XII. SUMMARY OF AMINO ACID SEQUENCING EXPERIMENTS.

SAMPLE	PROCEDURE	RESULTS
SCM 2.3 KD	1. HPLC GEL FILTRATION (3 CYCLES).....	SINGLE PEAK
	2. AFFINITY HPLC (ANTI-SCM mAb COLUMN)..	
	3. RP-HPLC C3.....	PROTEIN IN FLOWTHROUGH
	4. G10 DESALTING, SEQUENCING.....	DI-,TRIPEPTIDES: SAMPLE DEGRADATION
SCM 2.3 KD	1. HPLC GEL FILTRATION.....	SINGLE PEAK
	2. RP-HPLC C8 OR C18.....	PROTEIN IN FLOWTHROUGH
	3. G10 DESALTING, SEQUENCING.....	DI-,TRIPEPTIDES: SAMPLE DEGRADATION
SCM 4.7 KD	1. HPLC GEL FILTRATION (3 CYCLES).....	SINGLE PEAK
	2. RP-HPLC C8 OR C18.....	PROTEIN IN FLOWTHROUGH
	3. G10 DESALTING, SEQUENCING.....	DI-,TRIPEPTIDES: SAMPLE DEGRADATION
SCM 13 KD	1. AFFINITY HPLC (ANTI-SCM mAb COLUMN)..	
	2. HPLC GEL FILTRATION OF ELUATE.....	SINGLE PEAK, 13 KD
	3. RP-HPLC C3.....	SINGLE, BROAD PEAK
	4. RP-HPLC C18 OR C4.....	MULTIPLE LOW MOL. WT PEAKS
	5. SEQUENCE ONE PEAK (30 pM).....	MIXED/N-BLOCKED
SCM 13 KD	1. AFFINITY HPLC.....	
	2. RP-HPLC C18, C8 OR C4.....	SINGLE, BROAD PEAK
	3. SEQUENCING.....	DI-,TRIPEPTIDES: SAMPLE DEGRADATION
SCM 20 KD	1. SDS-PAGE PVDF MEMBRANE.....	
	2. SEQUENCING.....	N-BLOCKED
SCM 13-15 KD	1. SDS-PAGE PVDF MEMBRANE.....	
	2. SEQUENCING.....	N-BLOCKED
GBM 11 KD	1. AFFINITY HPLC (ANTI-SCM mAb COLUMN)..	
	2. HPLC GEL FILTRATION OF ELUATE.....	SINGLE PEAK, 11 KD
	3. RP-HPLC C18.....	BROAD PEAK
	4. RECYCLE C18, SEQUENCING.....	DI-,TRIPEPTIDES: SAMPLE DEGRADATION; N-BLOCKED
β-LACTOGLOBULIN CONTROL	1. SDS-PAGE PVDF & SEQUENCING.....	APPROPRIATE SEQUENCE
	2. RP-HPLC & SEQUENCING.....	APPROPRIATE SEQUENCE

DISCUSSION

The link between infection by specific group A streptococci and subsequent autoimmune disease has been described by several investigators (Futcher, 1940; Rammelkamp et al. 1952; Stollerman, 1969; McLaren et al. 1975; Nissenson, 1979). Evidence has been presented which supports an autoimmune/molecular mimicry model for the etiology of post-streptococcal disease (Markowitz and Lange, 1964; Lange et al. 1985, 1986; Fitzsimons et al. 1987). In support of this hypothesis, nephritogenic antibodies have been developed which are specific for streptococcal membrane-derived proteins (Fitzsimons et al. 1987). These proteins should share some immunochemical properties with the mammalian autoantigens, specifically with the GBM antigens recognized by anti-SCM antibody. As an initial step in characterizing the antigens in this autoimmune system, this dissertation describes the immunochemical properties of streptococcal cell membrane protein antigens recognized by an anti-SCM mAb cross-reactive with GBM. The results have implications for the structure of the SCM antigens and the immunologically related BM autoantigens. Furthermore, a model for APSGN can be constructed which accomodates these new observations regarding the SCM antigens and the monoclonal antibody.

Immunologically related antigens in SCM and BM

One aim of these studies was to isolate SCM protein antigens using this IgM anti-SCM mAb. Isolation of specific antigens was ac-

complished by affinity chromatography. Though several observations suggested that this anti-SCM IgM mAb bound its homologous antigen with relatively low affinity, affinity chromatographic isolation of immunologically related antigens was successful. Affinity isolation may have been facilitated by increased affinity associated with multivalent interactions between IgM and specific antigens (Hirayama et al. 1985; Poncet et al. 1988; Mallet et al. 1989).

Because immobilized IgM would have multiple paratopes available for antigen binding, antigen:Ab molar combining ratios of at least 1:1 were anticipated. The antigen:Ab combining ratios were 2:1 for SCM and 3:1 for LBM, but only 1:1 for GBM. Higher combining ratios were not observed possibly because of steric hindrance of paratopes. An alternative explanation is that multiple epitopes in each antigen occupied more than one paratope. The absolute number of epitopes for each antigen (antigen valences) cannot be determined from these data because antigen valence is a constant which is independent of the antigen:Ab combining ratios (Van Regenmortel, 1988). The relatively low antigen:Ab combining ratio for the GBM antigen may be due to masking of protein epitopes. GBM is composed of many glycoproteins with O-linked disaccharides and Asn N-linked heteropolysaccharides consisting of galactose, mannose, hexosamines, sialic acids and fucose (Spiro, 1967). GBM has a high hydroxylysine content (Spiro, 1967; Lange, 1969; Lange et al. 1986) and as much as 70% of these residues are involved in the linkage of the disaccharide units. Thus, these carbohydrates may interfere with interaction of the mAb with protein epitopes in GBM (Blue and Lange, 1975). Since

these GBM samples were digested with trypsin and not with carbohydrase or collagenase, carbohydrate masking remains a possibility.

Of note, each column bound only one major component from each membrane preparation (trypsin-digested SCM, GBM, LBM and SDS-solubilized SCM). How does one explain the selection of a specific sized antigen from fairly complicated membrane trypsin digests which contains smaller antigens? The concentration of the antigen in the digest certainly would affect the probability of its being selected. Though the absolute amount of each component in the digest is difficult to determine, from HPLC tracings of the SCM digest it appears that the larger fragments are a relatively higher proportion of the SCM trypsin digest. Another reason why predominantly one size antigen was bound from each digest could be related to the effect of antigen size on stability of epitopes. These antigens may be of the minimal size required to maintain an antigenic conformation which cannot be attained or stabilized in the smaller peptides from the digest (Sundaralingam et al. 1985; Gras-Masse et al. 1988). Larger antigens may have not been bound because of the restrictive environment of the column. That is, steric factors related to the column support material and the large size of IgM may permit only certain sized fragments to align and bind mAb with sufficient affinity (Bergold et al. 1988; Frenz and Howath, 1988). As a result, the immobilized IgM mAb may bind with higher affinity antigen having multiple epitopes or epitopes that are available due to the peptide's structural stability (Bergold et al. 1988; Frenz and Howath, 1988).

Support for the assertion that the antigenic peptides vary in their number of epitopes is the observation that mAb column-binding efficiencies of the tryptic SCM polypeptides when applied individually increased with increasing size. These observations suggest a mechanism by which the columns could selectively bind the larger antigens from the complete digest. Competition of antigens for IgM would favor antigens with more epitopes because such antigens would favor multivalent interactions with mAb (Bergold et al. 1988; Frenz and Howath, 1988). Taken together these experiments suggest that differences in binding between these polypeptides occurred, despite only small differences in size, because of differences in number or size-dependent stability of epitopes.

SCM antigens exhibit size-dependent reactivity

As described above, the SCM tryptic polypeptides not selectively bound by the affinity columns did bind when applied individually, but at different efficiencies. These antigens showed size-dependent reactivity in other experiments also. The unexpected result of routine screening of the low MW SCM fractions was that equal microgram amounts of select sized antigens were equally reactive in ELISA; their reactivities thus increased with size.

A parameter essential to the interpretation of this ELISA is how much polypeptide antigen is actually bound to the microtiter wells. Variation in plate binding efficiency during the coating step could be expected to affect the interpretation of epitope densities if it were determined that different polypeptides bound the

solid phase with different efficiencies. In solid phase assays the antigen concentration would seem difficult to determine since it can be expected that not all the antigen added to the system may actually bind to the solid phase. This ELISA used polypeptide concentrations of 2.5-10 ug/ml. Within this range the polypeptides showed 90% binding to the plates. It is not uncommon for proteins in this concentration range to bind nearly completely to plastic (Pesce et al. 1977) since plastic binding is by non-specific electrostatic attraction. Naturally, as antigen concentration increases, the actual fraction bound will decrease. That each antigen showed similar percent binding at the concentrations employed strengthens the argument that these polypeptides were present in equal amounts and thus differed in their molar reactivities with mAb.

Another problem with solid phase assays is the availability of epitopes when antigens are bound to plastic. This presumably would affect the reactivity of the smaller SCM antigens more than the larger antigens, which potentially have more epitopes. To overcome this problem in interpreting the solid-phase ELISA a competitive inhibition assay was designed in which the interaction of antigen with mAb could be assayed in liquid phase. Two different inhibition assays were developed. Low levels of inhibition were achieved by each antigen using the first method (mAb + inhibitor 1h 37^{o}C, then 18 h 4^{o}C). However, it was not possible to compare these antigens because of the relatively low levels of inhibition. It was reasoned that the mAb had low affinity for the liquid phase inhibitors,

allowing displacement of the inhibitor by the solid phase antigens. The mAb may prefer the orientation the antigen assumes while bound to solid phase. Though the proteins are in equilibrium among various folded and unfolded states while in solution, the plastic bound orientation would be stable. Other investigators have reported mAb which preferentially bind protein antigens which are solid phase. Djavadi-Ohaniance and coworkers (Djavadi-Ohaniance et al. 1984 and Friguet et al. 1984) reported a population of mAbs which bind the β_2 subunit of Escherichia coli tryptophan synthase in solid phase but are unable to interact well with the protein in solution. Apparently this population of mAbs binds determinants which are exposed or stabilized when the protein is bound to plastic.

The second assay utilizing solid phase C1q was developed to test whether solid-phase C1q could either enhance the stability of the mAb/inhibitor interaction or selectively remove inhibitor-bound antibody. Purified C1q (Yonemasu and Stroud, 1971) has been used in solid phase to detect circulating immune complexes (Hay et al. 1976; Wehler et al. 1981; Singh and Tingle, 1982; James et al. 1983). Certain antibodies bound to multivalent antigens will bind C1q efficiently (Brown and Koshland, 1975). Human IgG_1 and IgG_3 bind C1q strongly while IgG_4 binds only very weakly (Augner et al. 1971). In the murine system, IgG_{2a} binds C1q strongly (Leatherbarrow and Dwek, 1984) while IgM from all species binds C1q efficiently. This assay exploits the ability of C1q to bind IgM complexed with antigen.

The results clearly show the effect of antigen size on the amount required to inhibit mAb. Each antigen shows a dose-dependent

inhibition of mAb, but the 9.2 and 7.0 kd antigens cause relatively high levels of inhibition with relatively small amounts of antigen. This effect was not as extreme in the inhibition assay run without C1q. These results may be due to the unique interaction of C1q with IgM complexed with multivalent antigens. Swanson et al. (1988) showed the effect of antigen epitope density on the ability of C1q to bind IgM. C1q binding to IgM was significantly enhanced when IgM was complexed to multivalent antigens. Though IgM bound the different antigens with similar avidity, the binding of C1q to IgM was directly related to the epitope density of the antigens (Swanson et al., 1988). Their hypothesis was that C1q binding to IgM was enhanced because multivalent antigen binding caused a favorable conformational change in the C1q-binding region of IgM. Because the reactivity of the SCM antigens appeared to be related to molecular size, these antigens may vary in their number of epitopes. Thus larger 7.0 and 9.2 kd antigens with more epitopes would participate in multivalent IgM binding leading to efficient C1q binding. The net result would be more efficient removal of mAb when complexed to the higher epitope density antigens. Many more molecules of the smaller antigens would be required to reach similar levels of inhibition because their lower number of epitopes probably affects both mAb affinity and efficiency of C1q binding.

To confirm the cross-reactivity of the mAb with GBM in the ELISA system, inhibition of ELISA was performed. Of interest, only one nanogram (20 pmoles) of the 5 kd GBM fraction was able to inhibit binding of 12.5 pmoles mAb to SCM. Theoretically 1 pmole IgM

should bind on average 5 pmoles antigen but these results suggest that efficient inhibition occurred with only two-fold antigen, since 20 pmoles of the 5 kd fraction resulted in 95% inhibition of 12.5 pmoles antibody. In general, inhibition is probably not just dependent on paratope binding by the inhibiting antigen, but also on steric hindrance of unbound paratopes, so the size of the inhibitor is a critical factor. In a different study, Fitzsimons et al. (1987) inhibited anti-SCM mAb binding to SCM with 1 ng unfractionated GBM digest. In contrast, the present study utilized a purified low MW antigen; thus, the level of inhibition achieved by this 5 kd fraction is significant since it probably reflects specific binding to the paratope. Furthermore, the high levels of inhibition achieved by only 2-fold 5 kd antigen may reflect the occupancy of multiple paratopes by single antigens.

Monoclonal antibody affinity and valence are directly related to antigen size

The relationship between antigen size and mAb binding was further investigated. Analysis of mAb/antigen interactions in affinity chromatography suggested that the antigens bound with different efficiencies. ELISA and competitive inhibition experiments further suggested that antigen size was directly related to reactivity. The question arose whether the affinity of the mAb for each antigen was also related to antigen size.

Equilibrium dialysis determined that mAb/antigen affinity and mAb valence indeed varied with the size of the antigens. The non-

linear least squares regression analysis plots yielded K_d values varying from 10^{-5} to 10^{-7}, within the range of affinities reported for the interaction of IgM with multivalent haptens or antigens (Chua et al. 1975; Huchet and Feldman, 1973; Makela and Kontiainen, 1971; Makela et al. 1970). Of interest, affinities of the mAb for the antigens paralleled the increasing size and reactivities of these antigens. If these antigens do vary in their number of epitopes, it is possible that the increasing affinities are due to the the associated increased opportunities for multivalent interactions with mAb. Thus the observed functional affinity of IgM binding to antigen would be governed by the number of epitopes available.

The pronounced effect of antigen valence upon antibody affinity were described first for the interaction of a divalent antibody with multivalent antigens (Singer and Campbell, 1952 and 1953). The relationship between the intrinsic association constant (K_o, affinity of individual antibody combining sites for single epitopes) and the observed functional affinity was described by the following relationship:

$$K_M = nK_o/2$$

where K_M is the observed association constant of the interaction between a divalent antibody molecule and an antigen molecule of n number of combining sites. From this relationship, it is clear that the valence of the antigen molecule may exert a significant effect upon the overall affinity of the interaction even though the average intrinsic association is of low magnitude.

In another study of the interaction of divalent antibody with multivalent antigens, Larsson (1989) found a "bonus effect" due to divalent antibody binding. In this model an increase in the number of epitopes caused an increase in antibody binding and affinity that was much more than proportional to changes in epitope number (Larsson, 1989). Thus, small increases in epitope number could produce significant increases in binding affinity.

In studies of the interaction between IgM and multivalent antigens, the effect of multivalent binding upon functional affinity was also noted. It has been shown that the pentamer structure of IgM may confer a biologically active functional affinity even when the intrinsic affinity of individual combining sites is as low as 10^2 (Ternynck and Avrameas, 1986; Karush, 1978). A higher functional affinity of pentameric IgM mAb specific for hen egg lysozyme (HEL) was demonstrated by Mallet et al. (1989). Here, the interaction of pentameric anti-HEL mAb with HEL was not inhibitable by HEL in ELISA; monomeric mAb derived by reduction and alkylation of the pentamer was readily inhibitable. In this case the pentameric structure and resultant multivalent binding did not allow displacement of the bound antigen by the soluble inhibitor. Additionally, the pentameric form was polyreactive in ELISA, binding a variety of other proteins including self antigens, while the monomer was specific for HEL. Hence, the biologically active levels of reactivity (and poly- or autoreactivity) observed with pentameric IgM are thought to be due to multivalent antigen binding (Hirayama et al. 1985; Poncet et al. 1988; Mallet et al. 1989).

The mAb valence for the 9.2 kd is 2, while the mAb valence for the 7.0, 4.7, 2.3 kd is from 6 to 10. Of interest, mAb affinity was inversely related to mAb valence. This result is consistent with studies of the interaction between haptens and IgM where it has been found that IgM may have low valence and high affinity binding sites or high valence paired with low affinity binding sites (Pascual and Clem, 1988; Giles et al. 1983). The low valence found associated with high affinity in these experiments may indicate that the larger antigens have more epitopes than the smaller antigens. As discussed, the larger number of epitopes could promote higher affinity binding; at the same time, the larger number of epitopes could occupy more paratopes and thus prevent measurements of higher valence. However, calculation of antigen valence for these antigens according to the method described by Van Regenmortel (1988) for IgG Fab was not possible because of the complication introduced by intact multivalent IgM.

SCM antigens retain reactivity following heat-denaturing conditions

The binding characteristics of the different SCM polypeptide antigens in affinity chromatography and ELISA can be explained as the effect of increasing molecular size and complexity on the stability of antigenic conformations (Gras-Masse et al. 1988). An alternate interpretation is that the membrane antigen has repeating sequential epitopes, and that the polypeptides evaluated are fragments derived from this repeating epitope structure. The repeating epitope is not likely to be carbohydrate, since amino acid

analysis of SCM and the SCM fractions reveals no amino sugars. Furthermore, the molecular weights of the SCM fractions as determined by amino acid analysis agree with the molecular weights determined by HPLC, which also suggests that these are non-glycosylated polypeptides. The epitopes would most likely be defined by repeating polypeptide sequence or conformation.

Conformation may be negligible in these small polypeptides. In studies of secondary structure stability it was found that helix formation is at most negligible for short peptides (< 13 residues) (Sundaralingam et al. 1985). The stability of secondary structures and helices was strongly dependent on the number of residues capable of forming intrapeptide salt bridges. Thus, a minimal size as well as appropriate composition are required for short peptides to assume stable secondary structures (Padmanhabhan et al. 1990). If conformation is achieved by the SCM antigens in solution, the larger SCM antigens may be able to acquire more stable secondary structures than the smaller antigens. Existing conformation would then be disrupted by the denaturing effect of heating, yielding additional sequential determinants.

Heating proteins at 100^{o}C has been shown to cause irreversible denaturation (Ahern and Klibanov, 1985; Kenett et al. 1990). However, the disruption of conformation of some proteins has been shown to be reversible upon contact with antibody in solution. Appropriate binding conformation can be induced in a protein by antibody in solution (Sela, 1966; Djavadi-Ohaniance et al. 1984). But these polypeptides are coated on polystyrene plates where it may

be assumed that their conformation (whether native or denatured) remains fixed after coating. Several investigators have shown that proteins are denatured by coating to plastic or nitrocellulose membranes. mAbs which bind exclusively the solid phase form of tryptophan synthase do not bind the enzymatically active (native) protein in solution (Djaravadi-Ohaniance et al. 1984; Friguet et al. 1984). Furthermore, these mAbs can bind the denatured protein in solution, indicating that the solid phase protein is also denatured. Similar results were obtained for α-mannosidase (Mierendorf and Dimond, 1983), rat brain hexokinase (Finney et al. 1984), E. coli F_1-ATPase (Dunn et al. 1984), lactate dehydrogenase and creatinine kinase (Vaidya et al. 1985), bovine serum albumin (Smith and Wilson, 1986), and lysozyme (Kenett et al. 1990). Therefore, because the small size of the SCM polypeptides precludes significant secondary structure and because the denaturing conditions of boiling and plate coating eliminate existing secondary structure, the reactivity of these antigens is thought to be due to their primary amino acid structure.

Amino acid composition is related to antigen binding characteristics

Denaturing conditions did not destroy reactivity of these antigens, and in fact, appeared to increase reactivity. Since apparent N-terminal blocking prevented amino acid sequencing, amino acid analysis of the SCM antigens was performed to determine whether amino acid compositions could be related to these observations.

The MWs as determined by HPLC agreed with those obtained by

amino acid analysis, suggesting that these polypeptides are non-glycosylated and that the epitopes lie in the amino acid structure. These antigens showed a recurrence of particular amino acids, especially the relatively acidic amino acids and aliphatic side chain amino acids. Critical amino acids may be those which increase in number directly with antigen size; these include Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, and Leu. The most acidic, Glu and Asp, increased the most with antigen size, nearly doubling with each approximate double in antigen size. The relative acidity of these antigens paralleled the increase in antigen size, reactivity and mAb affinity for these antigens, suggesting a role for these amino acids in mAb recognition. Of note, these same amino acids were previously shown to be in abundance in immunologically-related peptides isolated from GBM (Lange, 1969).

These acidic amino acids are among the most hydrophilic and predictions of antigenic determinants based on hydropathicity profiles often show them as contributing to charged topographic epitopes (Hopp, 1986). A structural motif consisting of charged groups may be the minimal requirement for expression of an epitope (Edmundson et al. 1987; Payelle-Brogard et al. 1989; Stollar et al. 1989).

A proposed structure for the SCM antigens

The SCM antigens described here are related by their ability to bind a mAb cross-reactive with BM. Therefore these antigens have significant structural similarities. However, analysis of the interaction of these antigens with mAb shows that these antigens

vary in their reactivity and affinity. A structural basis for these differences was sought; amino acid analysis showed that the abundance of acidic amino acids was directly related to affinity and reactivity. When it was shown that these antigens were reactive following denaturing conditions, it was concluded that the amino acid sequence determined the expression of the epitopes. To accomodate all of these observations, a model for the structure of the SCM antigen is proposed in which the protein antigen's amino acid sequence contains a periodic distribution of acidic amino acid residues.

This model implies that these low MW polypeptides are antigenic subunits which can be derived from a larger antigen simply by proteolytic cleavage of the larger segment. Amino acid analysis showed that tryptic cleavage sites are available in the 20 and 13 kd SCM antigens; these sites may allow the generation of the smaller (11-2.3 kd) antigens. That some tryptic sites remain in these larger antigens suggests that trypsin digestion was incomplete. It has been shown that Arg or Lys which have at their C terminus an amino acid residue with anionic side chains (i.e. hydroxyl or carboxyl groups) are relatively resistant to trypsin cleavage (Smith et al. 1963; Canfield, 1963; Ambler and Brown, 1967). The abundance of Asp, Glu, Thr and Ser in these antigens may partially protect some potential trypsin sites.

The occurrence of repeating epitopes would give the smaller antigen fragments certain predictable characteristics. These include size-dependent differences in number of epitopes and resulting

differences in reactivity and affinity for the same antibody. Immunochemical analysis shows that the SCM antigens have these characteristics. Since structural analysis was limited to amino acid analysis, the confirmation of this model for the SCM antigen must rely on inferences from the amino acid composition data. That antigen size, affinity, and reactivity are correlated with the abundance of acidic amino acids suggests that these amino acids contribute to the epitope.

To describe the epitope as a "discontinuous linear array of acidic amino acids" is to describe a rather ill-defined epitope. Do such epitopes exist, and if so, do they have immunologically significant interactions with antibody? Recent studies have indicated that the occurrence of a linear array of charged amino acids can be quite sufficient for the expression of an epitope (Edmundson et al. 1987; Payelle-Brogard et al. 1989; Stollar et al. 1989). Such epitopes are often seen along the ridges of coiled proteins like collagen (Izui et al . 1976) or M protein (Gras-Masse et al. 1988). However, the SCM antigen may be defined by primary amino acid acid structure. Can a primary amino acid structure have these same characteristics? Recent studies were performed to identify the antigenic determinants of peptides derived from the breakdown of oncogene proteins (Appel et al. 1990). The major antigens were polypeptides of 13-30 residues and thus serve as a good point of reference for the SCM antigens. By synthesizing a series of peptides with single residue substitutions along the length of this peptide, it was possible to evaluate the contribution of each amino

acid to its antigenicity. The antigenic determinant was linear and about 4-6 residues in length. The amino acids found to be most important for the expression of the epitope were the aromatic amino acids and the charged hydrophilic amino acids and alanine. Furthermore, replacement of an aromatic with an aromatic or a hydrophilic with another hydrophilic did not affect antigenicity, suggesting that the determinant is defined by general characteristics of residues. Also, alanine could be substituted along the length of the polypeptide with little effect on antigenicity, suggesting that the precise sequence of residues is of secondary importance; though the determinant is linear, it is discontinuous. Thus the determinant is defined by spaced aromatic and charged amino acids (Appel et al. 1990). Finally, peptides with the intact determinant were able to interact with mAb in solution as measured by an inhibition ELISA, indicating that epitopes of this nature are quite capable of binding Ab and are thus of immunologic significance. Other investigators have demonstrated the existence of linear protein epitopes in influenza virus hemagglutinin defined simply by spaced amino acids (Jackson et al. 1986 and 1988; Schoofs et al. 1988).

These data have implications for the structure of the immunologically related basement membrane antigens (Markowitz and Lange, 1964; Fitzsimons et al. 1987). The immunologic relatedness of SCM and basement membrane antigens may be manifested in repeating epitopes in both membranes. Though the SCM epitope appears to be defined by the amino acid sequence, possibly by the repeating occur-

rence of acidic amino acids, the basement membrane epitope need not also be defined by amino acid sequence, despite similar amino acid compositions (Lange, 1969). The minimal requirement for the expression of the mammalian autoantigen may be a similarly distributed topography of charged groups resulting from protein conformation.

Low molecular weight antigens may have role in APSGN

These SCM antigens may have a role in the development of APSGN. The immune complex model for APSGN would suggest that low MW antigens induce the formation of circulating immune complexes which become trapped in the renal glomerulus (Michael et al. 1966), leading to the characteristic immunopathology seen in APSGN. The autoimmune/molecular mimicry model for APSGN would propose that SCM antigens bear epitopes common to BM and that the immune response to SCM antigens becomes directed at the mammalian antigens (Markowitz and Lange, 1964; Lange et al. 1985, 1986; Fitzsimons et al. 1987).

These data will be discussed first in the light of the immune complex model for APSGN. This model proposes that circulating streptococcal antigens combine with IgG and IgM to form immune complexes which deposit in the glomerular basement membrane (Michael et al. 1966). The immune complexes then activate complement, releasing vasoactive amines and chemotactic peptides. This results in increased permeability of glomerular capillaries and the recruitment of neutrophils and polymorphonuclear lymphocytes. Neutrophils attempt to phagocytose the immune complexes. When small complexes in subepithelial deposits cannot be phagocytosed, the

cells release their lysosomal enzymes which degrade the GBM. The GBM filtration capacity is destroyed and the individual develops acute renal failure. The recovery from acute nephritis occurs due to the eventual clearance of the circulating immune complexes. Phagocytic clearance may become more efficient due to the formation of larger immune complexes or because the antibody response matures to produce higher affinity antibody (Haakenstad and Mannik, 1977).

Do the observations from this thesis support this immune complex scenario? First, the low MW SCM polypeptides described here may contribute to the formation of circulating immune complexes that persist in circulation since immune complexes consisting of Ig and low MW antigens tend to remain small and thus persist in circulation longer (Haakenstad and Mannik, 1977). Second, the low affinity IgM mAb in this study may favor the formation of persistent immune complexes (Haakenstad and Mannik, 1977). Third, low MW immune complexes form subepithelial deposits while large immune complexes form endothelial deposits in the glomerulus (Haakenstad and Mannik, 1977). Subepithelial deposition of immune complexes is characteristic of APSGN, and has been observed in murine GBM following injection of this mAb (Lange, 1980a; Lange et al. 1985).

Though low MW antigens and low affinity antibody may have a role in the formation of immune complex-induced nephritis, a critical characteristic of this laboratory's animal model for APSGN is inconsistent with the immune complex model. This animal model supports the autoimmune/molecular mimicry model because the model shows that injection of this mAb alone is sufficient for inducing

nephritic syndrome in mice (Lange, 1980a; Lange et al. 1985, 1986). At no time do these animals receive streptococcal antigen. Furthermore, the mAb can be demonstrated in these animals' kidneys by immunofluorescence and can be eluted from kidney tissue (Lange 1980a; Lange et al. 1985). These observations suggest that the initiation of disease is due to cross-reactive antibody binding to specific kidney antigens.

This thesis also provides data concerning the chemical nature of SCM antigens which do not support the immune complex model. Immune complexes, antibodies, antigens, and other molecules which are cationic are preferentially trapped in the glomerulus because the GBM has a net negative charge (Rennke et al. 1975; Mannik et al. 1987). The SCM antigens were abundant in acidic amino acids. The pIs of serine, threonine, aspartic acid, and glutamic acid are 5.68, 6.53, 2.97, 3.22, respectively. Thus at physiological pH these amino acids would be negatively charged and would not be susceptible to entrapment in GBM.

How do these antigens trigger the autoimmune response? The immune response to streptococcal antigens during infection may be immediately cross-reactive with kidney antigen or it may become a cross-reactive response when other factors allow the expression of kidney autoantigens. In the first case, antibodies to streptococcal antigens bind immunologically-related GBM antigens which are readily available and presumably are expressed prior to infection. In the second case, streptococcal infection causes the expression of novel, modified, or previously sequestered antigens. In either case, it is

the similarity of bacterial and mammalian protein antigens that accounts for the cross-reactive immune response.

Bacterial protein antigens that are immunologically related to mammalian antigens have been linked to the development of other autoimmune diseases. Adjuvant arthritis has been induced in rats by a 65 kd antigen of Mycobacterium tuberculosis (van Eden et al., 1988). Adjuvant arthritis T-cell clones were used to screen genetically engineered truncated proteins and synthetic peptides derived from this 65 kd protein, and a nonapeptide from this antigen has been identified that is similar to a nonapeptide from the link protein of rat proteoglycan (van Eden et al., 1988). The shared amino acids may constitute a shared epitope, and therefore may be responsible for the development of autoreactive T-cells in adjuvant arthritis.

Ankylosing spondylitis (AS) and Reiter's syndrome (RS) are often cited as classic examples of molecular mimicry-associated autoimmune disease (Schwimmbeck et al. 1987). These non-rheumatoid arthritic diseases of unknown etiology have been associated with infections by Salmonella, Shigella, Yersinia, and especially Klebsiella pneumoniae. Schwimmbeck et al. (1987) reported that sera from HLA $B27^+$ AS and RS patients contained antibodies which recognized an epitope in K. pneumoniae nitrogenase and in HLA B27. The shared epitope was defined by six consecutive amino acids. Such a similarity would explain the high incidence of the HLA B27 haplotype within the AS and RS patient population. Other infectious agents have proteins that share stretches of consecutive amino acids

with specific mammalian proteins. Moreover, these common antigenic determinants are associated with the development of autoimmune disease: examples include streptococcal M protein and cardiac myosin in rheumatic heart disease (Krisher and Cunningham, 1985); adenovirus and A-gliadin in ulcerative colitis (Kagnoff et al. 1984); several viruses and myelin basic protein in experimental allergic encephalomyelitis (Dyrberg and Oldstone, 1986; Fujinami and Oldstone, 1986).

As in many infection-associated autoimmune diseases, the infectious organism can not be isolated from patients with APSGN (Rodriguez-Iturbe, 1984). This suggests that the autoimmune disease is initiated during infection and then perpetuated by the presence of autoantigens. It is proposed that the SCM antigens described in this thesis may have a specific role in the initiation and perpetuation of autoreactivity. Following infection, these antigens could bind low affinity antibody which is cross-reactive with GBM epitopes. This low affinity antibody could then bind exposed GBM epitopes; whether disease would be initiated at this point is not known. However, the antibody titer and affinity may mature because of the persistence of SCM antigens: the low affinity antibody binding and low molecular weight of the SCM antigens would favor the formation of small circulating immune complexes which tend to persist. Furthermore, persistence in circulation would be enhanced by the charge properties of these antigens. Maturation of antibody affinity and increase in titer could then lead to the critical density of antibody on GBM required to activate complement,

resulting in tissue damage. Such damage may then expose more GBM epitopes, leading to disease in the absence of streptococcal infection or antigens.

SUMMARY

The etiology of acute post-streptococcal glomerulonephritis, an autoimmune disease, may be explained in part by immunologic similarities between streptococcal membrane antigens and glomerular basement membrane. Because of this immunologic similarity, the protective immune response to streptococcal infection becomes destructive to host tissue. Support for this hypothesis is the observation that antisera and monoclonal antibodies to streptococcal membrane bind directly to glomerular basement membrane and cause disease in mice in a dose-dependent fashion.

This dissertation describes immunochemical properties of streptococcal cell membrane (SCM) protein antigens which were isolated using anti-SCM monoclonal antibody (mAb) cross-reactive with glomerular basement membrane (GBM).

Previously observed immunologic relatedness between SCM and basement membrane (BM) was confirmed by the isolation of a distinct antigen from each membrane by affinity chromatography with anti-SCM mAb. One major antigen was isolated from SCM, GBM, and lung basement membrane (LBM); these were 20 kd from SDS-solubilized SCM and 13, 11, 10 kd from trypsin-digested SCM, GBM, LBM, respectively. The antigen-mAb combining ratios on the affinity columns differed for each antigen and were found to be 2:1, 1:1, and 3:1 for SCM, GBM, LBM, respectively. These differences were thought to be due to differences in the antigens' relative number of epitopes.

Several low MW (11.6, 9.2, 7.0, 4.7, 2.3 kd) SCM antigens also present in the SCM trypsin digest but not selectively bound by the

affinity column were prepared by HPLC and subsequently found to be reactive with the mAb. These antigens bound the affinity column when applied individually, though they bound at different efficiencies. The % binding increased with the size of the individual antigen. Furthermore, these antigens were equally reactive with mAb when present in equal ug amounts in solid phase ELISA. Moreover, these SCM antigens inhibited mAb in a dose-dependent fashion with the degree of inhibition directly related to antigen size. Collectively, these results show that the reactivity of these antigens is related to their size.

The affinity of the mAb for the 9.2, 7.0, 4.7, 2.3 kd antigens was measured by equilibrium dialysis. The K_ds were directly related to antigen size and ranged from 10^{-7} to 10^{-5}. Valence of the mAb also varied for each antigen and was found to be inversely related to antigen size. The mAb valence was 2.0 for the 9.2 kd antigen and approximately 6.0 for the 7.0 kd. Saturation of mAb with the 4.7 and 2.3 kd antigens was difficult to achieve and thus the mAb valence for these antigens could only be estimated (6-10). These results show an increase in affinity of the mAb and a decrease in mAb valence with increasing antigen size. These observations are consistent with the observations that these antigens differ in their reactivities with antigen size. Increasing affinity with size of the antigen may be explained by the presence of multiple epitopes which permit multivalent and higher affinity interactions with mAb.

The structural basis for these observations was investigated. First, the SCM antigens were subjected to denaturing conditions and

were found to retain reactivity with the mAb. This suggests that the epitope is defined by the primary amino acid structure. Second, amino acid analysis of these antigens showed that the MW of each antigen agreed with that obtained by HPLC gel filtration, suggesting that the antigens are not glycosylated. Finally, the abundance of acidic amino acids (especially Glu, Asp, Thr, Ser) and aliphatic side chain amino acids (notably Ala, Val, Ile, Leu) was directly related to the previously observed reactivities and affinities. Taken together, these observations suggest that the epitope is defined by the linear amino acid structure of these polypeptides, possibly by the presence of acidic amino acids. Furthermore, to account for the changes in reactivity and affinity with antigen size, it is proposed that these antigens may contain epitopes composed of repeating units of these amino acids.

The amino acid composition of these antigens is distinctly different from other nephritis-associated streptococcal proteins and thus may be a novel antigen related to post-streptococcal glomerulonephritis.

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

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