EXPLORING THE BASIS OF ANTIBODY-MEDIATED PEPTIDE-CARBOHYDRATE CROSS-REACTIVITY

by

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B.Sc. Simon Fraser University (Canada), 1990

THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

in the Department of Chemistry

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Simon Fraser University

August 1997

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0-612-24314-1

Canada

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Degree: Ph.D.

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Abstract

A panel of closely-related, monoclonal antibodies (mAbs) directed against the cell-wall polysaccharide (CWPS) of Group A *Streptococcus* (GAS) was used to investigate the molecular basis of Ab-mediated peptide-carbohydrate cross-reactivity. The mAbs used had restricted variable gene usage, suggesting a shared mechanism of binding epitopes on the CWPS. Epitope mapping using synthetic fragments of the CWPS confirmed that the same epitope on the CWPS was recognized by these mAbs.

Each of the anti-CWPS mAbs was used to screen several phage-displayed peptide libraries; thus, an enormous variety of molecular shapes was tested for its ability to bind the anti-CWPS mAbs. Each of the anti-CWPS mAbs tested isolated cross-reactive peptides. Competition studies indicated that many of the peptides bound at or near the carbohydrate-binding site. Surprisingly, each mAb isolated peptides with chemically-distinct consensus-sequences which, in general, were able to distinguish between the closely related anti-CWPS mAbs. Similar results were obtained with three polyclonal antibodies directed against different synthetic oligosaccharide fragments of the streptococcal CWPS.

Cross-reactive peptides were also isolated by mAbs directed against carbohydrate structures unrelated to the CWPS of GAS. The mAbs Se155.4 and SYA/J6 which are directed against the O-antigens of *Salmonella* serogroup B and *Shigella flexneri* Y, respectively, both isolated unique cross-reactive peptide sequences. Thus, peptide cross-reaction with anti-carbohydrate antibodies has been demonstrated for seven mAbs and three polyclonal antibodies directed against three different carbohydrate structures. When

taken together with other recent published work, these results suggest that peptide cross-reaction with anti-carbohydrate antibodies is a general phenomenon.

The peptide sequences isolated by three of the anti-CWPS mAbs and SYA/J6 were chosen for further investigation. Peptides corresponding to the sequences isolated and analogous peptides containing amino acid-substitutions at defined positions were synthesized on solid supports using Chiron's Multipin™ peptide synthesis kit in order to determine the residues critical for recognition and the molecular requirements at these positions. Theses studies identified a SYA/J6-reactive peptide that was commercially synthesized in order to probe further its interaction with mAb.

Dedication

For Scot

No pithy saying, just my love.

Acknowledgments

The research reported in this thesis could not have been completed without the collaboration of many people. First and foremost, I would like to thank my co-supervisors, Drs. B. Mario Pinto and Jamie K. Scott, for providing the opportunity to do such interdisciplinary work. My gratitude extends to those laboratories outside Simon Fraser kind enough to allow me to work and learn with them; Dr. D. McNaughton and Mrs. J. Primmer, at Red Deer Regional Hospital, and Dr. D. R. Bundle and Ms. M. A. J. Gidney at the National Research Council. I would like to thank Ms. Gidney specifically for teaching me everything I needed to know about ELISAs and Dr. Bundle for his ongoing interest and support. A great deal of appreciation should be given to Dr. N. Greenspan; without his antibodies, this thesis would be a lot shorter.

I have had the good fortune to be associated with two outstanding research groups at Simon Fraser. I appreciate the support and conversation provided by all members, past and present, of the Pinto lab, and am very grateful to the entire Scott lab for making me part of their group. I would like to thank Ms. Lisa Craig for performing the initial biopanning experiments that started this entire project, Mr. Jarnail Mehroke and Mr. Mike Rashed for their technical expertise, Mr. Michael Zwick for his help with sequencing, Ms. Paula Brown for her help synthesizing ~~some of the~~ pin-displayed peptides, Drs. Jose Marino-Albernas and France-Isabelle Auzanneau for providing the various oligosaccharides, and Ms. Karla Randell and Mr. Doug Chou for finding time to read part of this thesis and to listen to me complain over coffee.

I am certain my parents did not know what to think when I started at the high school on the hill and then went into research, but they continue to support all my decisions along this journey. I am also certain that Scot did not know exactly what he signed up for when we started, but he too has been unfailing in his support and love. Lastly, I need to thank Mrs. Margaret McDermid for babysitting above and beyond the call of duty. If not for her, I would still be in front of the computer, writing this thesis, with Heather balanced on my knee.

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LIST OF ABBREVIATIONS

Abs	Antibodies
CWPS	Cell-wall polysaccharide
dGAS	Heat-killed pepsin treated Group A *Streptococcus*
D	Diversity gene of the antibody variable region
DIC	Diisopropylcarbodiimide
DMF	*N, N*-dimethylformamide
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
Fmoc	9-Fluorenylmethoxycarbonyl
Fab	Antigen-binding fragment of an antibody
GAS	Group A *Streptococcus*
Glc*N*Ac	*N*-acetylglucosamine
HIV	Human Immunodeficiency Virus
HOBt	Hydroxybenzotriazole
Id	Idiotypic
J	Joining gene of the antibody variable region
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline

pcAbs	Polyclonal antibodies
PS-SCL	Positional scanning synthetic combinatorial libraries
Rha	Rhamnose
SCL	Synthetic combinatorial libraries
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline
TRNOE	Transferred nuclear Overhauser effect
V	Variable gene of the antibody variable region

CHAPTER 1

INTRODUCTION AND BACKGROUND INFORMATION

1.1 Introduction to the Thesis

The work presented in this thesis is part of a program to develop potential immunodiagnostic reagents or vaccine targets for Group A *Streptococcus* (GAS). GAS is one of the primary infective agents in humans causing the relatively innocuous condition of streptococcal pharyngitis (strep throat), or more seriously, necrotizing fasciitis ("flesh-eating" disease) or toxic shock-like syndrome (1). Furthermore, GAS infections have been implicated as the trigger of autoimmune responses which lead to rheumatic fever or heart-valve disease (1). Our search for potential immunodiagnostic or vaccine targets was focused on the cell-wall polysaccharide (CWPS) of GAS. The structure of the CWPS is known and it shows little strain specificity; that is, its structure is the same for all strains of GAS (2). Both monoclonal antibodies (mAbs) and polyclonal antibodies (pcAbs) have been raised against the CWPS (3, 4, 5). However, there are disadvantages to carbohydrate based reagents. In general, carbohydrate reagents are expensive to synthesize and they are T-cell independent antigens (6). For the case of GAS in particular, immunization with carbohydrates may trigger an autoimmune response, since it has been demonstrated that Abs directed against the CWPS of GAS can bind different host tissues (1). The ability of anti-GAS CWPS Abs to bind host tissues is interesting; the Abs cannot be binding host tissues displaying the same carbohydrate structures present on GAS since mammalian systems do not contain rhamnose, one of the major

components of the GAS CWPS (2). Consequently, this autoimmune reaction may be due to protein-carbohydrate cross-reactivity.

Data from several studies suggest that Abs directed against the CWPS of GAS may bind protein structures on heart tissue. Kaplan was the first to demonstrate that the immunization of rabbits with streptococcal cell-walls elicited an immune response that bound cardiac tissue (7). Later studies showed that anti-streptococcal Abs recognized a variety of host tissues, from skeletal and smooth muscle to cardiac valves and epithelium of skin, and implicated the CWPS of GAS as an inducer of the autoimmune Abs (8). Several groups have demonstrated that Abs recognizing the immunodominant carbohydrate of the CWPS, Glc*N*Ac, also bind cardiac valves, epithelium and other host antigens (9). Furthermore, elevated levels of Abs directed against Glc*N*Ac are present during the acute phase of streptococcal infection and post-streptococcal sequelae (10).

Shikhman *et al.* (11, 12) recently studied the cross-reactivity of Abs directed against Glc*N*Ac. The Abs studied were raised against either GAS and shown to recognize Glc*N*Ac or a synthetic glycoconjugate of Glc*N*Ac-BSA directly. This work demonstrated that Abs capable of binding Glc*N*Ac also bound many cytoskeletal and heart proteins such as actin, keratin and myosin as well as streptococcal recombinant M5 and M6 proteins. Moreover, the cross-reactive epitopes recognized by these Abs were resistant to *N*-acetylglucosaminidase and sodium periodate treatment which suggests that these were non-carbohydrate epitopes, and most likely proteins. In order to analyze the nature of the cross-reactive epitopes on proteins, the binding of a Glc*N*Ac-reactive mAb to synthetic peptides was investigated. A peptide from coxsackievirus, which is known to

have a high level of homology with a protein (M6) on the streptococcal surface, was shown to bind an anti-Glc*N*Ac mAb. Through the use of synthetic peptides corresponding to short overlapping peptide fragments of the coxsackievirus peptide, Shikhman *et al.* (11) demonstrated that this Glc*N*Ac-reactive mAb recognized the amino acid sequence RRKLEFF.

Protein-carbohydrate cross-reactivity has been demonstrated in other bacterial systems, as well, using Abs directed against the V domains (i.e. anti-idiotypic (anti-Id) Abs) of anti-carbohydrate Abs. For example, Stein and Soderstrom (13), Monafo *et al.* (14), Hastings *et al.* (15), Evans *et al.* (16), Westerink *et al.* (17), and Furuya *et al.* (18) have all reported the binding of an anti-Id Ab to an anti-carbohydrate mAb. In some instances, a new carbohydrate-binding response was elicited when an anti-Id Ab was used as the immunogen (13-15, 17, 18). Anti-Id Abs that are able to elicit an immune response that binds the original antigen are said to contain an internal image of the original antigen; that is, the anti-Id Ab and the original antigen are believed to be chemically similar (19).

Recently, there have been reports of peptides cross-reacting with carbohydrates (Table 1.1). Hoess *et al.* (20) reported the isolation of a peptide from a phage-displayed library, which bound a mAb specific for the Le^y antigen; Bianchi *et al.* (21) isolated a peptide that bound a mAb specific for the core oligosaccharide of the lipopolysaccharide from *Shigella flexneri* A; Valadon *et al.* (22) isolated peptides that bound mAbs directed against the capsular polysaccharide of *Cyptococcus neoformans*. Westerink *et al.* (23) used the sequence of the CDR3 of an anti-Id Ab raised against a mAb specific for the

capsular polysaccharide of *Meningococcus* group C to design a peptide that cross-reacted with the capsular polysaccharide.

Table 1.1. Examples of antibody-mediated peptide-carbohydrate cross-reactivity.

Carbohydrate antigen	Sequences of cross-reactive peptide
Le^y antigen (20)	APWLYGPA
core oligosaccharide of the LPS from *Shigella flexneri* A (21)	HF(V/S)Q(H/R/G)[a]
capsular polysaccharide of *Cyptococcus neoformans* (22)	TPXW(M/L)(M/L)[a,b], (W/Y)XW(M/L)YE[a,b], XDWXDW[b]
capsular polysaccharide of *Meningococcus* group C (23)	CARIYYRYDGFAY

a) single letter abbreviations in parentheses indicate that more than one amino acid was found at a position
b) X represents a position occupied by random amino acids

Three of the cross-reactive peptides reported (see Table 1.1) contain an **arXar** motif (where **ar** is an aromatic amino acid): WLY in the Le^y-cross-reactive peptide (20), (W/Y)XY in the *Cyptococcus neoformans*-cross-reactive peptide (22), and YRY in the *Meningococcus* group C-cross-reactive peptide (23). Moreover, a lectin (24, 25) and a carbohydrate-reactive enzyme (26) have been shown to bind peptides containing the sequence YPY and yRy, respectively, where y represents D-tyrosine. These reports suggest that a specific peptide structure, in particular one containing aromatic amino acids, is required for polysaccharide mimicry (16). However, all of these reports used only one mAb, lectin or enzyme to screen peptide-libraries for cross-reactive sequences. Our work with seven anti-dGAS mAbs, three pcAbs, and two anti-lipopolysaccharide mAbs suggests that the **arXar** motif seen in the other published reports is probably not a

general carbohydrate-mimic motif, since in our work, only a few peptides isolated from the pcAb screens had this motif. Moreover, no other functional, shared motif was observed. It may be that the aromatic rich motifs isolated by other groups reflects an advantage provided by aromatic rings for interactions with proteins, rather than a structural mimicking of carbohydrates.

Attempts have been made to use cross-reactive peptides as immunogens to elicit carbohydrate binding responses. In one case, the peptide was shown to elicit Abs having the same idiotype as the cognate anti-carbohydrate mAb (22), and in another, to elicit a carbohydrate-binding response (23). Thus, the mechanism of Ab-mediated cross-reaction between peptides and carbohydrates remained to be established.

The work in this thesis addresses the generality of peptide-binding anti-carbohydrate mAbs since nine mAbs and three pcAbs were used in this study, and begins to address the molecular basis of Ab-mediated cross-reactivity between carbohydrate and protein antigens. The goal was to determine if the cross-reactive peptides recognized by anti-carbohydrate Abs would bind by the same mechanism as the corresponding epitope on the carbohydrate target. If so, the basis of cross-reactivity would be structural mimicry. Taking advantage of the fact that many anti-carbohydrate Ab responses have restricted variable (V)-gene usage, and thus recognize a single epitope on a carbohydrate antigen (22, 27, 28), we assembled a panel of closely-related mAbs against the CWPS of GAS, and showed, by oligosaccharide mapping studies, that they indeed bind a similar, if not identical, epitope (Chapter 2). These mAbs were then used to isolate cross-reactive peptides from a panel of eleven phage-displayed libraries, and a unique consensus

sequence was derived from the peptides isolated by most of the mAbs (Chapter 3). Each consensus sequence group was chemically distinct, as the peptide sequences were different between groups but very similar within groups. Moreover, representative peptides from each group were functionally specific, since they usually bound only their isolating mAb. Restricted peptide specificity that did not follow carbohydrate-reactivity was also observed for three pcAbs directed against synthetic oligosaccharide fragments of the GAS CWPS. Thus, the predominating basis of peptide recognition by anti-carbohydrate Abs differs between Abs, with true carbohydrate mimics being relatively rare. These data provide strong evidence for the hypothesis that peptide recognition is idiotype-specific (i.e., specific to an individual mAb). Thus, peptides provide a higher degree of discrimination between closely related anti-carbohydrate mAbs than do native carbohydrate haptens (29).

These results contrast sharply with the peptide cross-reactivity patterns observed with other sets of closely-related Abs that recognize protein epitopes (30). We propose that the antigenic mimicry observed for carbohydrate-cross-reactive peptides (and, by extension, anti-Id Abs) is determined mainly by the binding sites of anti-carbohydrate Abs (including small differences between them), rather than by chemical similarity to the corresponding carbohydrate epitope. This has significant implications for the role of peptides as immunogen mimics in eliciting carbohydrate-reactive Ab responses since a given peptide mimic may not elicit the spectrum of reactivities present in the response against the corresponding carbohydrate immunogen. Moreover, peptides would probably

perform poorly as surrogate antigens in place of carbohydrates, as they would only be recognized by a subset of the reactivities in an anti-carbohydrate response.

Short discussions on the general nature of Abs and peptide libraries, produced either chemically or biologically using phage-display, are provided in this introductory chapter. Following this background information, Chapter 2 provides a description of the anti-carbohydrate Abs used in this thesis.

Chapter 3 describes the work performed to determine if the cross-reactive peptides recognized by anti-carbohydrate Abs would bind by the same mechanism as the native carbohydrate epitope. This work involved a panel of closely-related mAbs directed against the CWPS of GAS which allowed us to begin investigating the mechanisms involved in Ab-mediated peptide-carbohydrate cross-reactivity. All of the mAbs used for this work isolated cross-reactive peptides, and a unique consensus sequence, which was functionally specific for the isolating mAb, could be derived from the peptides isolated by most of the mAbs. Restricted peptide specificity that did not follow carbohydrate reactivity was also observed for three polyclonal Abs directed against synthetic oligosaccharide fragments of the GAS CWPS. Thus, the predominating basis of peptide recognition by anti-carbohydrate Abs differs between Abs, with true carbohydrate mimics being relatively rare.

Chapter 4 presents investigations of the role of individual amino acids of two cross-reactive peptides. Chiron's Multipin™ peptide synthesis kit was used to synthesize many analogs of the cross-reactive peptide sequences (31-35). The first question was whether the peptides would be recognized when isolated from the large coat-protein of

the phage. Consequently, the cross-reactive peptide sequences were synthesized first; these peptides were recognized by their corresponding mAb. Next, the residues critical for peptide binding were determined by studying various analogs of several of the parent peptides containing amino acid substitutions. A residue was determined to be important for recognition if mAb binding was significantly decreased or abolished upon substitution by another amino acid.

Finally, Chapter 5 pertains to the characterization of glycoconjugate affinity reagents made from two different matrices, either the 3M Emphase™ Biosupport Medium AB 1 (UltraLink™ from Pierce, Rockford, IL) or PEGA resins which are beaded polyethyleneglycol dimethylacrylamide co-polymers synthesized in our laboratory. These affinity reagents were prepared by Dr. France-Isabelle Auzanneau using two different oligosaccharides corresponding to portions of the CWPS of GAS (36, 37). The affinity reagents were used to purify anti-oligosaccharide Abs from polyclonal sera. The efficiency of the UltraLink™ affinity reagents and the PEGA affinity reagents were compared.

1.2 Antibody Structure and Function

There are many elements to the immune system that work together to eliminate infectious agents. Abs are just one element of this well-adapted system (38). A great deal of the work in this thesis involves Abs. Thus, a brief introduction to the important features of Abs follows.

Abs are glycoproteins, found free in the blood or lymph, all of which possess the same basic structure. As shown in Figure 1.1, Abs are "Y"-shaped molecules composed of two distinct types of polypeptide chains, one of which is heavier than the other (38, 39). Each "Y"-shaped molecule has two heavy chains and two light chains held together by disulfide bonds; however, in a given Ab, both heavy chains are identical as are both light chains. Each binding site of an Ab is formed by one light chain and one heavy chain. Thus, most Abs are bivalent and can bind the same molecule, called an antigen, at both binding sites (38).

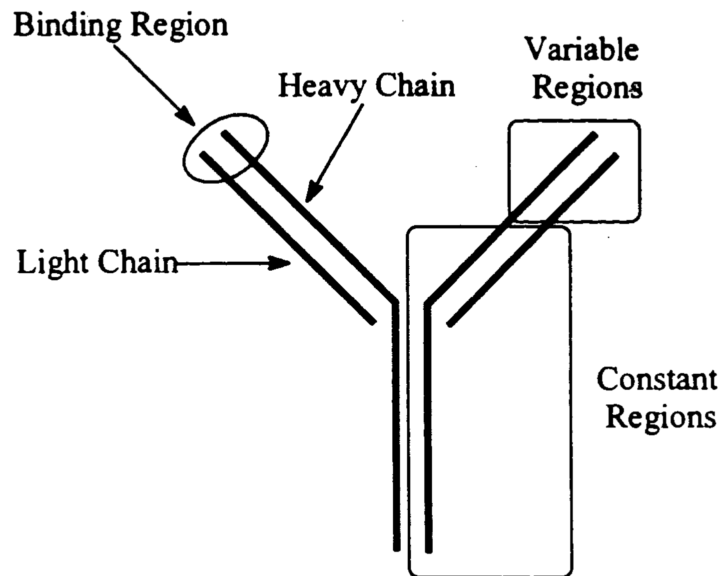


Figure 1.1 Schematic representation of an antibody molecule

In most higher mammals there are five different types of heavy chains, α, δ, ε, γ, and μ, and two types of light chains, κ and λ (38, 39). Each class of heavy chain can combine with either class of light chain. It is the use of different heavy chains that leads to different classes, or isotypes, of Abs (38, 39). The five Ab isotypes corresponding to the different heavy chains are IgA, IgD, IgE, IgG, and IgM, respectively. All the isotypes

are composed of the same basic "Y"-shape; for instance, as shown in Figure 1.2, IgGs are composed of a single bivalent "Y"-unit, while IgMs are composed of five identical bivalent "Y"-units resulting in ten identical binding sites.

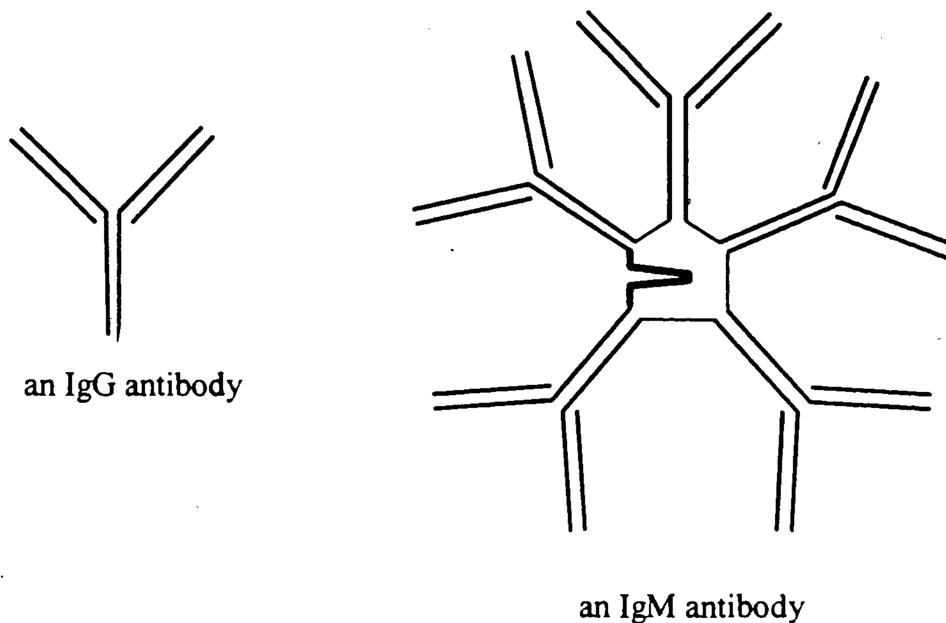


Figure 1.2 Schematic representation of an IgG antibody and an IgM antibody

Variation within a heavy chain type gives rise to subclasses. For example, there are four subclasses of IgGs in humans, IgG1 through IgG4 (38). Other differences between Abs can be classified as allotypic or idiotypic (38, 39). Allotypic differences refer to differences between Abs of the same isotype within a species. While every member of a species will possess all the isotypes of its species, different members will possess different allotypes due to genetic variability between individuals. Idiotypic differences refer to differences between Abs of the same isotype and allotype within one individual

(40). These differences are found in the variable regions of Abs since it is these regions that vary among the Abs made by an individual.

It is possible to raise Abs that recognize the idiotype of an Ab; such Abs are called anti-idiotypic (anti-Id) Abs (40). Jerne proposed that anti-Id Abs formed an Ab network. The original Ab, or Ab1, would be generated in response to a foreign body, the antigen. The Ab1, in turn, would generate a response, Ab2, since its variable region would be foreign. The Ab2s can be classified into different subtypes: Ab2α whose binding to the Ab1 is not inhibitable by the original antigen, Ab2β whose binding to the Ab1 is inhibitable by the original antigen and, when used as an immunogen, generates an immune response (Ab3) that recognizes the original antigen, and finally Ab2γ whose binding to the Ab1 is also inhibitable by the original antigen but does not produce an Ab3 response that recognizes the original antigen (Figure 1.3) (41, 42). Ab2β are said to contain an "internal image" of the original antigen, that is the Ab2β combining site may mimic the original antigen and thus, produces an Ab3 that recognizes the original antigen.

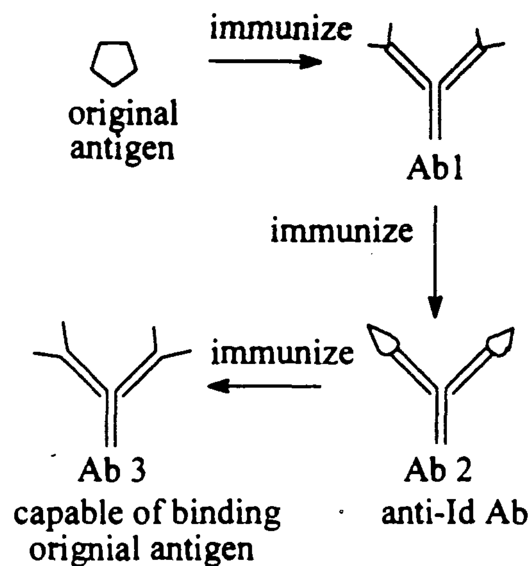


Figure 1.3 Schematic representation of an antibody network.

Anti-Id Abs are generally directed against the Variable (V)-regions of an Ab which comprise the *N*-terminal domains formed by the light and heavy chains. The amino acid sequences of the V-regions vary greatly among Abs; however, these differences are not scattered throughout the entire region; rather, they are concentrated within three regions of hypervariability. These regions of high sequence variability are known as the complementarity determining regions (CDRs) since they are responsible for most of the contacts made with antigens. The CDRs form solvent exposed loops (hypervariable loops), well positioned to bind antigens. The regions of higher sequence homology that separate the CDRs are known as the framework regions. The sequences of the *C*-terminal domains of the light and heavy chains are less variable than the *N*-terminal domains and are, therefore, referred to as the constant regions.

Whereas the sequences of the hypervariable loops differ, analysis of known Ab structures has revealed that these regions form loops with only a small number of main-

chain conformations or canonical structures (43-46). A canonical structure is determined by the loop size and the types of amino acids at key positions within the loop and framework regions. Canonical structures have been identified for all of the hypervariable loops of the light chain, but for only two of the hypervariable loops of the heavy chain. The third hypervariable loop of the heavy chain is the most variable region among Abs since it results from the genetic recombination of three different gene segments (47). Consequently, its sequence, and even length, are extremely variable (48) and canonical structures have not been identified. The extreme variability of this region is significant since the third hypervariable loop of the heavy chain is found in the center of the binding pocket and thus plays a major role in Ab specificity (43).

When an Ab does interact with an antigen, many non-covalent interactions are formed in the interface between an Ab and its epitope (39, 49). An epitope is the small portion of an antigen which interacts with an Ab combining site. Generally, epitopes on protein antigens are discontinuous, that is, the residues on the antigen making high energy contacts with an Ab are located in distant regions, and are brought together by folding of the polypeptide chain. The major interactions between an Ab and its epitope are hydrogen bonds, electrostatic interactions, van der Waals interactions and hydrophobic forces (39, 49). These interactions mainly involve the side chains of Ab amino acids although the protein backbone sometimes contributes (39, 49). The aromatic amino acids are capable of forming many of the interactions involved in Ab-antigen complexes and are more likely to be found in the CDRs than in the framework regions (39, 50-52). Aliphatic amino acids are also involved in forming key interactions with bound antigens.

The concentration of aromatic amino acids in Ab combining sites has been observed for the many protein-binding Abs and the few carbohydrate-binding Abs studied to date. In contrast, lectins, which are carbohydrate-binding proteins, do not have as many aromatic amino acids in their carbohydrate-binding pockets, but rather have a preponderance of charged residues (91).

Water plays an important role in the formation of Ab:antigen complexes (54). Initially, the unliganded Ab and antigen are dissolved in an aqueous environment, thus the polar groups on the surface are hydrated and the entire molecule is surrounded by a network of hydrogen-bonded water molecules. Water molecules that are directly bonded to the polar groups have restricted motion compared to those in the bulk solution; the water molecules over the nonpolar patches are highly ordered while those over the polyamphiphilic patches are perturbed, or unordered. These solvation shells must be broken, and the water released to the bulk solution, in order to form the Ab:antigen complex. The release of the water molecules from the polar groups, as well as those over the hydrophobic surfaces, leads to an increase in entropy. In contrast, the return of water from the polyamphiphilic patches to the bulk solution decreases the entropy (54). Thus, the restructuring of water released from solvation shells provides an important force for the formation of biological complexes (54).

Another way in which water plays a role in biological complexes is to facilitate the formation of some hydrogen-bond networks. Sometimes water molecules in the Ab combining site contribute extra hydrogen bonds to the Ab:antigen complex (39). The presence of bound water can improve the complementarity between an Ab and its antigen

by increasing the number of non-covalent interactions and the overall stability of the complex. Some Ab combining sites have been shown to change their conformation upon binding an antigen (39, 55-57). Such induced fit mechanisms can increase the complementarity between the combining site and the antigen and result in a more enthalpically favorable interaction.

Although individually the non-covalent interactions that govern Ab:antigen interactions are weak, when they act together, a high affinity can be achieved (39, 49). The affinity of an Ab for its antigen is defined as the affinity of one binding site for its monovalent antigen. However, most Ab:antigen interactions are not monovalent since Abs have two or more binding sites and many antigens are multivalent. This multiplicity of interaction results in a binding energy that is considerably greater than the sum of the affinities since all of the Ab:antigen interactions must be broken simultaneously in order to destroy the Ab:antigen complex. This multiplicity of binding is termed avidity (49). Moreover, since the individual interactions between an Ab and its epitope are weak and distance dependent, the surfaces of both must be highly complementary (49). Abs recognize the overall conformation of an epitope rather than just specific groups (i.e., the correct chemical groups must be presented in the correct spatial arrangement). As a result, Ab:antigen interactions are quite specific; Abs can distinguish small differences in amino acid sequence, as well as differences in charge or shape. Consequently, most Abs will only bind native protein antigen; once an antigen has been denatured, it may no longer be bound by the Ab. Thus, Abs raised against whole proteins often do not bind peptide portions of the protein and conversely, Abs raised against peptides often do not

bind the native protein. However, if two antigens present similar surfaces, whether they are from the same chemical family or not, one Ab may bind both antigens. In such a case, the antigens are said to cross-react. In order to investigate the possible cross-reactivity of carbohydrates and peptides, we investigated well characterized anti-GAS CWPS mAbs and polyclonal antibodies (pcAbs) raised against synthetic portions of the GAS CWPS, as well as two other well characterized anti-carbohydrate mAbs.

1.3 Introduction to large and small peptide libraries

A peptide library is a collection of peptides with different sequences. Large peptide libraries contain millions to ten billion different sequences, and therefore, can contain all, or close to all, the amino acid combinations possible for a given length. It is possible to use a binding protein (e.g., Abs) to affinity purify a few tight-binding peptides from the large excess of nonbinding peptides. Therefore, hundreds of millions of different sequences can be screened at one time, thus peptide libraries offer a rapid means of discovering novel, biologically active peptides. Small peptide libraries do not contain as many different sequences and were used in this thesis to study, to the level of a single amino acid, the lead peptide sequences found from screening the large phage-displayed libraries. Whether large or small, library technology represents a significant advance in the study of biologically relevant peptides.

1.3.1 Phage-displayed peptide libraries

Biological approaches have been used to make large peptide libraries. Parmley and Smith developed a filamentous bacteriophage expression vector that could display foreign peptides on its surface (58). This technology allowed the construction of vast collections of random peptide sequences and the selection of those peptides capable of binding a receptor (59-62). Since the libraries contain a significant portion of all the possible peptide sequences of a given length, no *a priori* knowledge of a protein's sequence is needed. The essential feature of phage-displayed libraries is that each peptide is synthesized *in vivo* and is associated with its coding sequence in the phage. Thus, when phage with binding peptides are affinity purified, the coding sequence is also obtained and can be used for subsequent amplification and identification of the peptide.

The filamentous bacteriophage used to display the libraries are single stranded DNA phage that infect male *Escherichia coli* cells through the F pilus (for reviews of phage biology and morphogenesis see references 63-65). The phage are long flexible rods consisting of a protein coat surrounding a single stranded DNA. The protein coat is composed of one major coat protein, cpVIII, and several minor coat proteins, including cpIII. Approximately 2700 cpVIII molecules are assembled to form the tubular array that covers the single stranded DNA; in contrast, there are only three to five copies of cpIII all clustered at one end of the phage. The cpIII is necessary for infecting bacterial cells. At the beginning of infection, the cpIII interacts with a receptor on the end of the F pilus which triggers the pilus to retract. Through unknown mechanisms, the single stranded DNA is injected into the bacterial cytoplasm where it is quickly converted to double

stranded DNA and then a double stranded replicative form. The replicative form is the template for mRNA transcription and for production of progeny single stranded DNA. The newly synthesized viral proteins move to the inner membrane where they are stored until viral assembly occurs. Importantly, viral assembly of these filamentous phage does not occur in the cytoplasm; rather, by processes that are not well understood, the virions are assembled and extruded from the inner membrane. Thus, the bacterial host cells are not killed by this phage infection but growth is significantly slowed resulting in fuzzy plaque formation. Therefore, if the phage contain antibiotic resistance markers, infected bacterial cells can be selected and grown on antibiotic plates.

Both cpIII and cpVIII have been used for peptide display (Figure 1.4) (59-62, 66-68). The first libraries were displayed on cpIII (60-62). The cpIII is synthesized with an *N*-terminal signal peptide that is removed by signal peptidase after translocation through the inner membrane. cpIII is placed in the protein coat such that its *N*-terminal domain is solvent exposed; this domain mediates phage attachment to the F pilus. Smith's group demonstrated that fusing peptides near the *N*-terminus of cpIII did not destroy phage infectivity (58, 69); much evidence has been collected which suggests that most peptide sequences of less than one hundred residues can be fused to cpIII without affecting phage stability or infectivity (60-62). The peptide sequences displayed on cpIII used in this thesis are flanked by ADGA on their N-terminal side and GAAGA on their C-terminal side (60). These latter flanking sequences are intended to be structureless linkers to decrease the influence of the large cpIII upon the conformation of the displayed peptide.

Since all the cpIIIs are found at one end of the phage, cpIII-display results in multivalent binding.

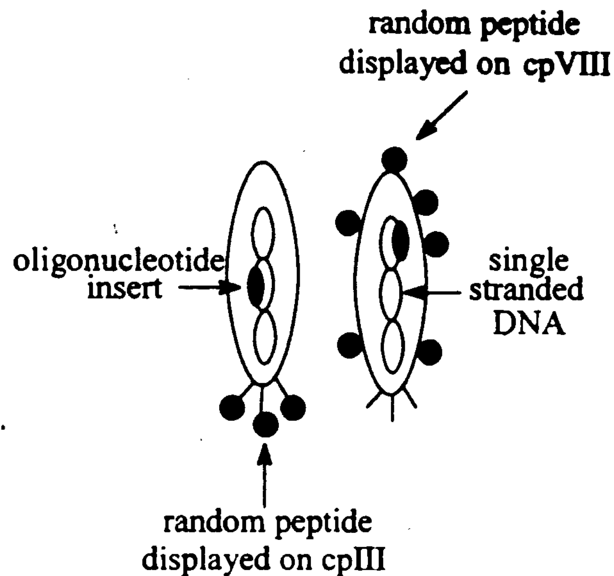


Figure 1.4. Schematic representation of phage-displayed peptides.

When peptides are displayed as fusions to cpVIII, more peptide copies will be displayed (Figure 1.4) (59, 66-68). cpVIII is also placed in the protein coat such that its *N*-terminal domain is solvent exposed. However, the phage will not be assembled if a peptide longer than five amino acids is fused to every copy of cpVIII (67, 68). This problem can be overcome by supplying a wild-type cpVIII (67, 69); thus, both wild-type and fusion cpVIII are used to assemble the virion. While libraries displayed on cpVIII potentially contain more copies of the peptides than cpIII-displayed libraries, the cpVIII fusion proteins are diluted with wild-type cpVIII and thus, monovalent binding is possible if the density of the fusion protein on the surface of the phage is low.

For either cpIII or cpVIII display, degenerate oligonucleotides are synthesized and cloned into appropriate sites within the single stranded DNA (59, 60, 66, 70). The oligonucleotides are synthesized with single nucleotides at positions encoding the invariant amino acids flanking the variable regions and equimolar mixtures of the nucleotides at positions encoding random amino acids. Therefore, the resulting library contains each amino acid represented proportionally to the number of triplets that encode an amino acid. For example, there is only one codon for methionine, but six for serine, therefore, serine would be found six times more often than methionine. This biological bias can be reduced by using NNK codons in the positions encoding the random amino acids, where N represents an equimolar mixture of all the nucleotides, and K represents an equimolar mixture of the nucleotides guanine and thymine. The resulting thirty-two possible codons encode all twenty amino acids but only one stop codon (60, 66, 70).

In order to contain all 64 million (20^6) possible hexapeptides encoded for by the approximately one billion (32^6) different hexacodons, a library would have to contain over a billion phage clones. In general, the libraries constructed have contained approximately 10^8 to 10^9 independent clones; 10^{10} clones may be the practical limit (66). One way to increase the diversity of hexapeptide sequences in a library is to construct libraries with larger variable regions (e.g. 15-mers). While it is not possible to express every possible 15-mer sequence, a larger diversity of short sequences will be represented in a 200-million-clone 15-mer library than in a similar sized library of shorter sequences (66).

In order to isolate binding peptides from the nonbinding background, receptor molecules, such as Abs, are used for affinity purification (58, 60, 70). The affinity purification, called biopanning, involves capturing Ab:phage complexes. The capture is accomplished by immobilizing a biotinylated Ab on a streptavidin coated support. The biotin-avidin interaction is one of the strongest non-covalent interactions known (71). An aliquot of the phage-displayed library is added, unbound phage are washed away and bound phage are then eluted with acid. The harsh elution conditions do not harm the infectivity of the phage; therefore, the eluted phage can be amplified by growth in *E. coli* cells. The amplified phage are used in further rounds of biopanning. The amplification procedure does introduce a biological bias to the system; some inserts may slow the growth rate of the phage, thus introducing a growth selection on top of the affinity selection. Nonetheless, after several rounds of biopanning, enrichment for binding sequences usually occurs. Individual phage clones can be isolated for characterization and DNA sequencing in order to determine the displayed sequence. The displayed sequences can be compared and the occurrence of similar sequences among independent clones is an indication of selective enrichment.

Binding peptides have been successfully isolated from phage-displayed peptide libraries using both monoclonal and polyclonal antibodies directed against peptides (30, 60, 62, 67, 72). For example, Scott and Smith were able to isolate the sequence DFLEKI using two monoclonal antibodies specific for this epitope of myohemerythrin (60). Therefore, phage-displayed peptide libraries can be used for epitope mapping, even when the protein sequence is not known. Moreover, the sequence CRFVWC, which bears no

resemblance to the DFLEKI epitope, was also isolated. Thus, it is possible to isolate novel binding peptides from phage-displayed libraries. One potential application of phage-displayed libraries is the discovery of mimotope peptides (72). However, antibodies directed against whole proteins have not been too successful in isolating tight binding peptides (30). Interestingly, binding peptides have been isolated by a few non-protein-binding receptors using both phage-displayed libraries and other library techniques (20, 22-26, 29, 61, 73). For instance, Devlin *et al.* (61) used streptavidin to isolate peptides that mimicked biotin, while Oldenburg *et al.* (24), Scott *et al.* (25), Etzkorn *et al.* (73), Westerink *et al.* (23), Hoess *et al.* (20), Bianchi *et al.* (21), Eichler *et al.* (26), and Valadon *et al.* (22) recently isolated peptides which appeared to mimic carbohydrates. The work in this thesis addresses the mechanisms involved in the Ab-mediated cross-reaction between peptides and carbohydrates (29). In order to determine the residues critical for the cross-reactivity, small peptide libraries based upon the lead peptide sequences discovered from the large phage-displayed peptide libraries were studied.

1.3.2 Small peptide libraries and Geysen's pin technology

Different chemical approaches have been used to prepare small peptide libraries (74, 75). The tea bag method developed by Houghten places the individual resins used for the solid-phase synthesis of various peptides into separate solvent-permeable packets, or "tea-bags" (76, 77). The tea-bags are treated all together for their common deprotection, washing and neutralization steps, but are placed separately into reaction

vessels containing different amino acids for each coupling reaction. This method generates multiple milligram quantities of hundreds of different non-support-bound peptides.

The tea bag method has been used to make synthetic combinatorial libraries (SCL) and positional scanning synthetic combinatorial libraries (PS-SCL) both of which can be screened for peptides with biological activity (74). One SCL was composed of 400 different peptide mixtures represented by $Ac\text{-}O_1O_2XXXX\text{-}NH_2$, where O represents a fixed amino acid and X represents a mixture of all the amino acids. (Four hundred different peptide mixtures are necessary since every amino acid is used for both fixed positions.) In order to determine the sequences of the active components, an iterative synthesis and screening process was performed in which one more fixed position was introduced each time. Consequently, several syntheses and screens were necessary. The PS-SCL use a different approach. For this approach, a large collection of different small libraries is initially synthesized but screened only one time. A PS-SCL is represented by the formulas $Ac\text{-}O_1XXXXX\text{-}NH_2$, $AcXO_2XXXX\text{-}NH_2$, $AcXXO_3XXX\text{-}NH_2$, $AcXXXO_4XX\text{-}NH_2$, $AcXXXXO_5X\text{-}NH_2$, and $AcXXXXXO_6\text{-}NH_2$. As a result, an active peptide sequence can be determined upon a single screening of all these mixtures.

Another chemical approach to making small libraries developed by Geysen (31-33) results in microgram quantities of tens of thousands different peptides covalently linked to the ends of different polyethylene rods. The peptides generated are tested while attached to the pins.

A method for generating mimotopes using the pin technology has been proposed (78). Mimotopes are peptides which mimic epitopes that are formed when different portions of a protein are brought together, in space, due to folding. Briefly, each pin contained thousands of octapeptides, each with two positions defined and six positions as mixtures (i.e., Ac-XXO_1O_2XXXX-pin, where O represents a defined position and X represents a mixture of amino acids). The 400 different pins each have different defined amino acids. Unlike the PS-SCL which are screened only once (74), an iterative synthesis and screening process, in which one more fixed position was introduced each time, is necessary to define a mimotope. As a result, Geysen's pin technology has found more use for the systematic mapping of linear epitopes within a known protein sequence (32-34, 75). The pin technology was used in this thesis to investigate the known cross-reactive peptide sequences discovered by screening large phage-displayed peptide libraries with carbohydrate-reactive mAbs.

In order to define precisely the epitopes to the level of a single amino acid, sets of peptides, whose sequences are based upon a known sequences, are synthesized (33, 35, 75). The principal method for determining the sequence of epitopes in proteins is to generate a complete set of overlapping peptides of a given length which are homologous with the sequence of interest (Figure 1.5) (33, 75). The peptides are then assayed for their ability to bind antibodies in order to determine which sequences represent epitopes. The length of the peptides must be equal to or greater than the longest linear epitope; generally, epitopes range from five to eight amino acids (79, 80); therefore, octapeptides should be used if all linear epitopes are to be found (33). Once an antibody-binding

peptide sequence is known, different sets of peptides can be used for the precise definition of the epitope to the level of a single amino acid (33, 35, 75). For instance, the minimum size of an antibody-binding sequence and the role of each amino acid in a sequence can be determined. The size of an antibody-binding peptide can be determined by a set of overlapping peptides with different lengths (Figure 1.6) (33, 75). The role of individual amino acids in the binding peptides can be determined by a replacement, or substitution, set of peptides which is generated by replacing each amino acid, one at a time, by every other amino acid (Figure 1.7) (33, 75, 79). Using such sets of peptides, the epitopes of myohemerythrin, foot-and-mouth disease virus, and myoglobin, for example, have been precisely defined (32, 34, 35, 81).

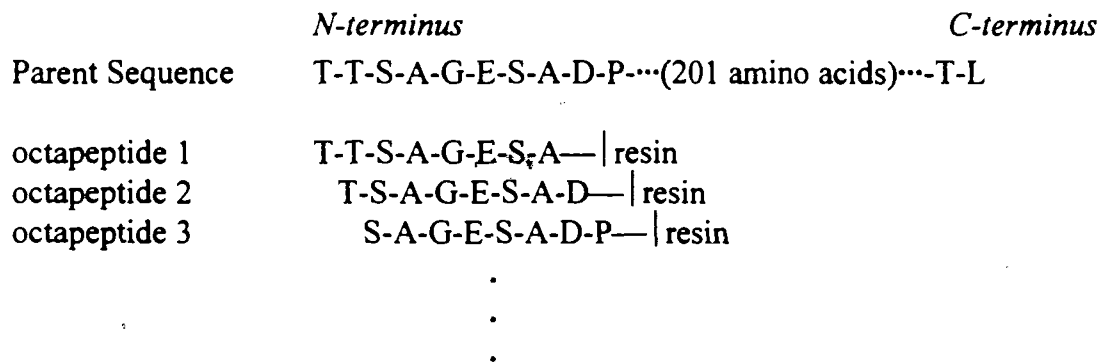


Figure 1.5. Schematic representation of the set of peptides generated in order to scan a protein sequence for epitopes. The parent sequence is from the viral protein 1 of foot-and-mouth disease virus (32).

Parent Sequence	···AIIHVLHSRHPGDFGADAQGAM···
	⋮
4-mers	GDFG DFGA FGAD
	⋮
5-mers	GDFGA DFGAD FGADA
	⋮
6-mers	GDFGAD DFGADA FGADAQ
	⋮
7-mers	GDFGADA DFGADAQ FGADAQG

Figure 1.6. A set of multiple length peptides which could be used to determine the size of an antibody-binding peptide, as well as the contribution to activity of the amino acids flanking the core. The parent sequence is from sperm whale myoglobin (81).

Parent Peptide	DFGAD		
	AFGAD	DAGAD	...
	CFGAD	DCGAD	...
	DFGAD	DDGAD	...
	EFGAD	DEGAD	...
	FFGAD	DFGAD	...
	GFGAD	DGGAD	...
	HFGAD	DHGAD	...
	IFGAD	DIGAD	...
	KFGAD	DKGAD	...
	LFGAD	DLGAD	...
	MFGAD	DMGAD	...
	NFGAD	DNGAD	...
	PFGAD	DPGAD	...
	QFGAD	DQGAD	...
	RFGAD	DRGAD	...
	SFGAD	DSGAD	...
	TFGAD	DTGAD	...
	VFGAD	DVGAD	...
	WFGAD	DWGAD	...
	YFGAD	DYGAD	...

Figure 1.7. A replacement set of peptides used to determine the role of individual amino acids within the epitope. The parent peptide is an epitope from sperm whale myoglobin (81).

The pin technology takes advantage of the common washing steps used for the entire set of peptides with only the specific amino acid being added requiring individual treatment (31-33) (Figure 1.8). Each pin is placed into an eight by twelve array; thus, the entire block of pins can be washed in bulk solvent baths or placed such that each pin sits in its own microwell containing a specific amino acid derivative or mixtures there of. It is, therefore, possible to synthesize hundreds of peptides simultaneously. The support-

bound peptides can be assayed several times; bound antibody is removed by washing the block of pins in a bath containing sodium dodecyl sulfate (SDS) and mercaptoethanol (31). Unfortunately, each different antibody must be tested separately which can be time consuming.

Using the different sets of peptides outlined above, epitopes of a known protein can be defined precisely to the level of a single amino acid. Moreover, a peptide with higher affinity may even be found among a replacement set of peptides (82-84). In this thesis, the pin technology was used to determine the residues critical for peptide binding to carbohydrate-reactive mAbs, as well as to begin investigating the chemical requirements at these positions.

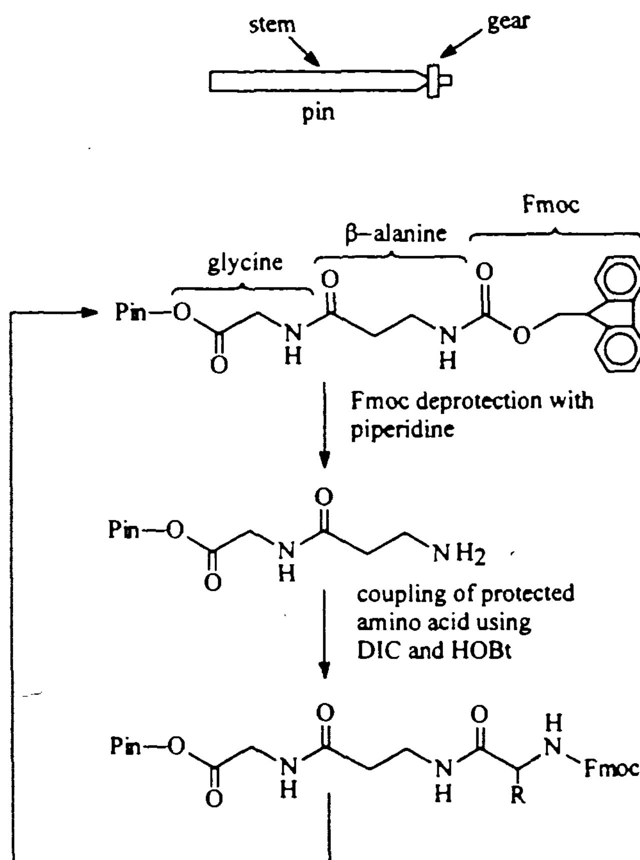


Figure 1.8. Schematic representation of the synthesis of peptides on pins.

CHAPTER 2

THE ANTI-CARBOHYDRATE MONOCLONAL ANTIBODIES AND POLYCLONAL ANTIBODIES

2.1 The anti-dGAS mAbs

We assembled a panel of eight closely related mAbs, SA-2 (3), SA-3 (3), SA-4 (3), HGAC 39 (4), HGAC 47 (4), HGAC 85 (4), HGAC 101 (4) and Strep 9 (5). The SA mAbs were IgMs and the HGAC mAbs and Strep 9 were IgG3s. All of the mAbs were raised in mice against a heat-killed, pepsin treated GAS (dGAS) whole cell vaccine (85). The pepsin treatment removed the capsular protein antigens to expose the cell-wall polysaccharide (CWPS) of GAS. The CWPS is composed of a poly-α-L-rhamnopyranosyl (Rha) backbone with branching *N*-acetyl-D-glucopyranosylamine (Glc*N*Ac) residues and is shown in Figure 2.1 (2). The Rha residues are joined with alternating α(1-2), and α(1-3) linkages, whereas the Glc*N*Ac branches are attached with β(1-3) linkages to the free 3-OH of alternating Rha residues.

The purification of all the mAbs from ascitic fluid has been described previously, (3, 4, 5) but only the SA mAbs were purified in our laboratory. The SA mAbs were precipitated from ascitic fluid by ammonium sulfate and dialyzed against PBS. No further purification was performed.

The sequences of the anti-dGAS mAbs are known for all the mAbs on the panel except SA-2 and SA-4 and are shown in Figures 2.2a and 2.2b. As for other anti-carbohydrate responses (22, 86, 87), the V_H-gene usage for the anti-dGAS mAbs is highly

restricted; identical germline V_H genes are used for all five mAbs. The tighter-binding mAbs, SA-3, Strep 9 and HGAC 39, all use the same V_H and J_H germline genes, and they also have the same number of residues in their CDR3s, as compared to the weaker-binding mAbs HGAC 47 and HGAC 101, which use different J_H genes and have relatively shorter or longer CDR3s. Together, the oligosaccharide mapping studies (see below) and the V_H and J_H gene usage suggest that the anti-dGAS mAbs recognize similar, if not identical, epitopes. Most likely, carbohydrate binding is mainly determined by V_H, since most of the sequence differences between the heavy chains are within CDR3, and V_L gene usage is relatively promiscuous. As will be shown later, SA-3, HGAC 39, and Strep 9 (and HGAC 85) form a group of higher-affinity anti-dGAS mAbs that share similar carbohydrate binding specificities. Since these mAbs would be expected to have the most similar sequences, it is surprising that those of Strep 9 and HGAC 85 differ from those of SA-3 by 25 and 28 residues, respectively, whereas HGAC 39 differs from SA-3 by only five.

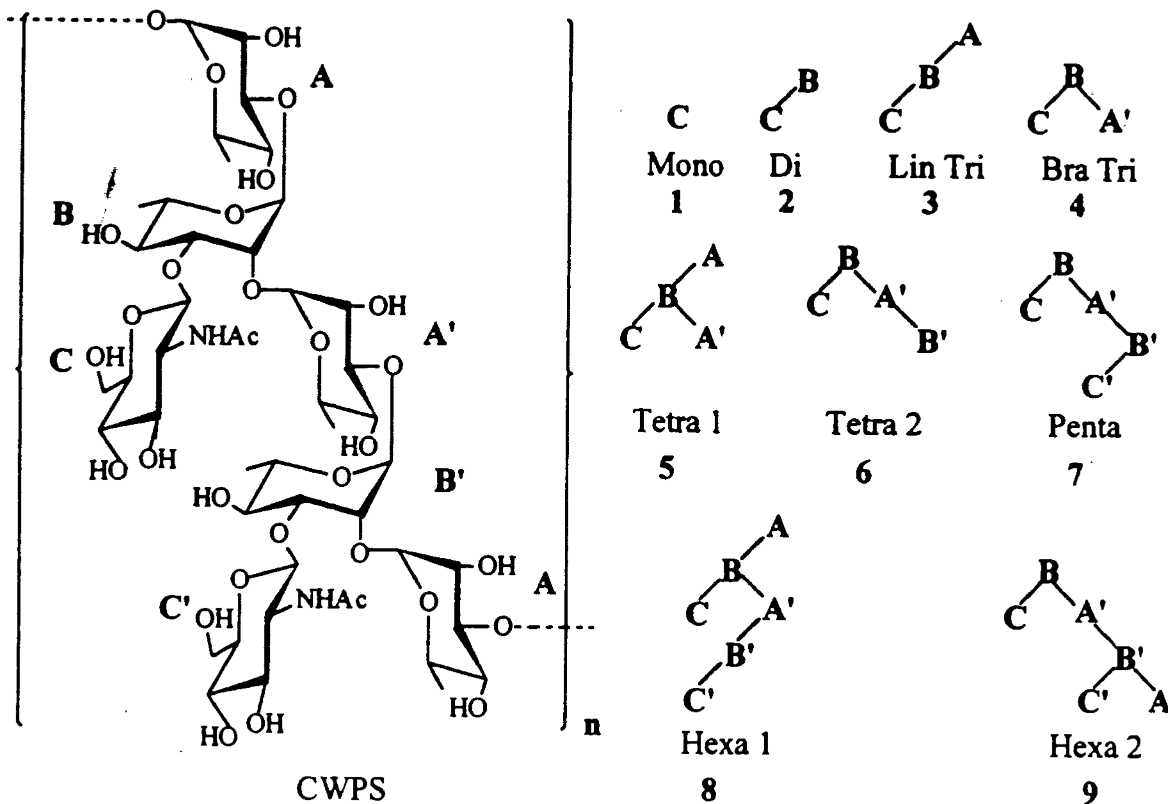


Figure 2.1. The CWPS of GAS and synthetic oligosaccharides corresponding to portions (structures **1-9**) of the CWPS.

V_H DOMAIN

```
                 FR1                      CDR1    FR2
SA-3      EVKLEESGGGLVQPGGSMKLSCVASGFTFSNYWMDWVRQSPEKGLEWVA
HGAC 39   -------------------------------------------------
Strep9    ----L-----------------F--------------------------
HGAC 47   -------------------------------H-----------------
HGAC 101  -------------------------------------------------
HGAC 85   -------------------------------H-----------------

SYA/J6    ---V------------------------------E--------------
Se155.4   --QVQQ--TV-AR----VKM--K---Y--T----H-IK-R-GQ----IG
```

```
            CDR2                       FR3
SA-3      EIRLKSNNYATHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGIYYCID
HGAC 39   ------D-F------------------------------------------
Strep9    Q-----D------------------------------------I-----TS
HGAC 47   -------------------------------------------------T-
HGAC 101  -------------------------------------------------T-
HGAC 85   ----Q------N----------------------------T--------T-

SYA/J6    -------------------------------------------------TR
Se155.4   A-YPGNSATFYNHKFRA-TKL-AVTSTITAYME-SSLTNEDSAVYYCTRGG
```

	CDR3 FR4	Gene Usage	
SA-3	LA...WFAYWGQGTLVTVSA	V_H 39	J_H 3
HGAC 39	-S...--------------	V_H 39	J_H 3
Strep9	P-...--P------T---EFA	V_H 39	J_H 3
HGAC 47	-GNYA.MD------S----S	V_H 39	J_H 4
HGAC 101	-G....MD------S----	V_H 39	J_H 4
HGAC 85	I-AT....------------	V_H 39	J_H 3
SYA/J6	GGAVGAMD------S----S		
Se155.4	HGYYG..D----AS--VS--K		

Figure 2.2a. Sequences and gene usage of the anti-dGAS mAbs, Se155.4 and SYA/J6 heavy chains (4, 29, 88).

V_L DOMAIN

	FR1 *CDR1* *FR2*
SA-3	DIVMTQAAFSNPVTLGTSASISCRSSKNLLHSNGITYLYWYLQRPGQSPQLLI
HGAC 39	------------------------------------F----------------
Strep9	---------------------------S---------------K--------V
HGAC 47	---------------------------S---------------K---------
HGAC 101	-V----TPLTLS--I-QP-----K--QS--D-D-K---N-L--------KR--
HGAC 85	--------T-E---P-E-V--------S--Q---N-----F------------
SYA/J6	-V-L--TPL-L--R--DQ--------QS----D-N---H----------K---
Se155.4	QA-V--ESALTTSPGE-VTLT.----TGTVTSGNHANWVQEKPDHLFTGLIGD

	FR3 *CDR3*
SA-3	YRVSNLASGVPNRFSGSESGTDFTLRISRVEAEDVGVYYCAQLLELPYT
HGAC 39	---
Strep9	-QM-V------D---S-G-----A------------------N------
HGAC 47	-QM--------D---S-G------------------------N----F-
HGAC 101	-L--~~K-D~~----D--T--G-------K--------L-----W-GPQF---
HGAC 85	--M--------D-----G---A------------------M-H--Y-V-
SYA/J6	-K---RF----D-----G-------K--------L---F-S-TTHV-.-
Se155.4	TNNRAPGVPARF..---LI-DKAA-T-TGAQP--EAI-F--LWCNNHWI

	FR4	Gene Usage	
SA-3	FGGGTKLEIK	Vκ 25-39	Jκ 2
HGAC 39	---------R	Vκ 25-39	Jκ 2
Strep9	---------R	Vκ 25-47	Jκ 2
HGAC 47	--X-	Vκ 25-47	Jκ 4
HGAC 101	---------R	Vκ 2 -91	Jκ 2
HGAC 85	--A------RA	Vκ 24A-8	Jκ 5
SYA/J6	----------		
Se155.4	------TVLGQPK		

Figure 2.2b. Sequences and gene usage of the anti-dGAS mAbs, Se155.4 and SYA/J6 light chains (4, 29, 88).

The minimal epitope on the CWPS recognized by the anti-dGAS mAbs was deduced by competition ELISAs using a panel of synthetic oligosaccharides comprising di- to hexa-saccharide portions of the CWPS, shown in Figure 2.1 (3, 36, 89, 90). None

of the carbohydrate inhibitors was able to inhibit fifty percent of the binding of SA-2 or SA-4 to an immobilized antigen. Thus, no conclusions as to the carbohydrate binding specificities of these mAbs could be drawn. However, the data in Table 2.1 show that the carbohydrates did inhibit all the other mAbs from binding immobilized dGAS. The mAbs could be separated into two groups: SA-3, Strep 9, HGAC 39, and HGAC 85, which bound the carbohydrates relatively tightly, and the weaker-binding mAbs, HGAC 47 and HGAC 101. The tighter-binding mAbs were best inhibited by the larger structures containing a branch point (CBA'). A branch point is defined as a sugar residue with two other sugar residues attached at non-anomeric positions. The branched trisaccharide (Bra Tri, **4**, Figure 2.1), which bears this minimal epitope, was a significantly better inhibitor for all of the mAbs than was the linear trisaccharide (Lin Tri, **3**). Furthermore, since the disaccharide (**2**) was a better inhibitor than the Lin Tri (**3**), a reducing α(1-3)-linked Rha residue probably introduces unfavorable interactions between the carbohydrate and the mAb. An extended carbohydrate surface also appears to be important for mAb recognition, since the tight-binding mAbs were best inhibited by all of the carbohydrates larger than a tetrasaccharide, as well as by purified CWPS. In contrast, the pentasaccharide (Penta, **7**) was the only large structure that significantly inhibited the weak-binding mAbs, HGAC 47 and HGAC 101. Thus, the branch point, and to a lesser extent, an extended surface are essential features of the epitope recognized by all of the anti-dGAS mAbs (3, 29).

Table 2.1. Affinity constants of synthetic oligosaccharides for the anti-dGAS mAbs.

	Hapten									
mAb	Mono	Di	Lin Tri	Bra Tri	Tetra 1	Tetra 2	Penta	Hexa 1	Hexa 2	CWPS[a]
SA-3	0.1	2.4	0.6	5.6	7.0	3.8	3.6	4.0	3.3 (5.71 ± 0.49)[b]	3.6
Strep 9	0.2	0.3	<0.1	0.7	0.6(1.5 ± 0.3)	2.0	2.7	1.6	3.7 (3.09 ± 0.67)	10.4
HGAC 39	n/d[c]	0.4	0.1	0.9	n/d	2.1	2.3	n/d	1.7	n/d
HGAC 85	n/d	0.5	0.7	0.9	n/d	2.3	2.8	n/d	1.8	
HGAC 47	n/d	0.1	<0.1	0.1	n/d	0.1	1.3	n/d	0.1	n/d
HGAC 101	n/d	0.2	<0.1	0.2	n/d	<0.1	0.3	n/d	<0.1	n/d

Affinity constants are the inverse of IC_{50} values determined by competition ELISA against immobilized dGAS; The values reported are measured in $10^4\ M^{-1}$. The error in the values determined by ELISA is approximately 10%.

a) the CWPS isolated and purified from GAS (89)

b) data in parentheses were determined by titration microcalorimetry

c) not determined

The binding of two of the anti-dGAS mAbs to oligosaccharides was also investigated by titration microcalorimetry by Dr. D. Bundle at the University of Alberta and Dr. B. Pitner, Becton Dickinson Research Centre. The data in Table 2.2, show that the interactions between SA-3 and Strep 9 and the oligosaccharides are weak compared to Ab:protein interactions. SA-3 and Strep 9 have similar affinities for Hexa 2 (**9**), while Strep 9's affinity for Tetra 1 (**5**) is of the same order of magnitude. All of these mAb:carbohydrate complexes have favorable enthalpies of formation that are offset by unfavorable entropies. These data are typical of carbohydrate:protein interactions in which a strong enthalpic contribution is offset by an opposing entropic contribution (91).

Table 2.2. Thermodynamic parameters of Strep 9 and SA-3 binding to oligosaccharides.

Strep 9	Tetra 1 (**5**)	Hexa 2 (**9**)
K_a (M^{-1})	$(1.5 \pm 0.3) \times 10^4$	$(3.09 \pm 0.67) \times 10^4$
ΔH (kcal/mol)	-5.8 ± 1.0	-12.02 ± 0.41
ΔG (kcal/mol)	-5.70 ● 0.4	-6.14 ± 0.05
TΔS (kcal/mol)	-0.02 ± 1.0	-5.86 ± 0.41

SA-3	Hexa 2 (**9**)
K_a (M^{-1})	(5.7 ● 0.5) x 10^4
ΔG (kcal/mol)	-6.49 ± 0.05
ΔH (kcal/mol)	-5.62 ● 0.38
TΔS (kcal/mol	0.87 ± 0.43

The Bra Tri (**4**) has been shown to be a conformationally-restricted feature of the GAS carbohydrates (92-94). Dr. T. Weimar, a postdoctoral fellow in Dr. B. Pinto's laboratory, used transferred nuclear Overhauser effect (TRNOE) experiments to study the structural and dynamic behavior of a carbohydrate bound by the anti-dGAS mAb, Strep 9

(92). The Bra Tri (**4**) was selected for use since it represents the minimal epitope recognized by the anti-dGAS mAbs. Strep 9 was chosen as a representative mAb from the panel since it is an IgG Ab and a homology model has been constructed (5). Using rotating-frame Overhauser spectroscopy in conjunction with molecular mechanics, molecular dynamics (93) and Metropolis Monte Carlo calculations (94) the conformations of the Bra Tri (**4**) free in solution were determined. This combined experimental-computational methodology allowed the minimum energy conformations of the two glycosidic linkages to be defined for the Bra Tri (**4**) free in solution. The TRNOE studies of the Bra Tri (**4**)-Strep 9 complex illustrated that Strep 9 selected a conformation which is close to a local minimum energy conformation for the uncomplexed Bra Tri (**4**). A change from the calculated and observed global minimum conformation for the free Bra Tri (**4**) is a rotation about the α(1-2) bond that changes the ψ angle from + *gauche* to - *gauche*. We infer that the other mAbs in the panel also bind similar conformations within these conformational families since all the anti-dGAS mAbs show similar binding behavior with the carbohydrates and have similar sequences.

2.2 The anti-CWPS pcAbs

The generation and isolation of IgG pcAbs directed against synthetic portions of the GAS CWPS have been previously described (3). Briefly, three groups of rabbits were immunized with bovine serum albumin (BSA)-glycoconjugates of the Lin Tri (**3**), Bra Tri (**4**) or Penta (**7**) (see Figure 2.1). The IgG fractions of the sera were isolated by chromatography on a Protein-A column. The anti-BSA activity of the IgG fractions was

reduced by using a BSA-Sepharose matrix for an affinity column. The anti-carbohydrate Abs passed through the column while the anti-BSA Abs were retained.

The specificities of the pcAbs were shown to be similar to those of the anti-dGAS mAbs, in that the anti-Lin Tri and anti-Bra Tri pcAbs each bound only their cognate trisaccharide and Penta (7), whereas anti-Penta pcAb bound all three haptens (3); moreover, the data in Table 2.3 demonstrate that dGAS was bound best by the anti-Penta pcAb.

Table 2.3. Binding of pcAbs to dGAS

pcAbs	dGAS	BSA
anti-Lin Tri pcAb	250[a]	26
anti-Bra Tri pcAb	231	35
anti-Penta pcAb	653	39

a) $(A_{405}-A_{490})$ x 1000

2.3 The anti-*Salmonella* serogroup B mAb Se155.4

The IgG1 mAb, Se155.4, was raised against *Salmonella* serogroup B and is directed against the *O*-antigen of the lipopolysaccharide (LPS), which consists of a repeating branched unit, 4-*O*-(3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-2-*O*-α-D-galactopyranosyl-α-D-mannopyranosyl)-α-L-rhamnopyranose, shown in Figure 2.3 (88, 95).

The V_L sequence of Se155.4, shown in Figures 2.2a and 2.2b, has somatic mutations from the V_L germ line gene (88). These mutations imply that affinity-driven maturation modified the binding site during the immune response resulting in a relatively

tight-binding anti-carbohydrate mAb. Importantly, the sequence of Se155.4 is different from those of the anti-dGAS mAbs, and Se155.4 does not bind dGAS (Table 2.4).

Table 2.4. Binding of Se155.4 and SYA/J6 to dGAS

mAb	dGAS	BSA
Se155.4	28[a]	15
SYA/J6	30	31

a) $(A_{405}-A_{490}) \times 1000$

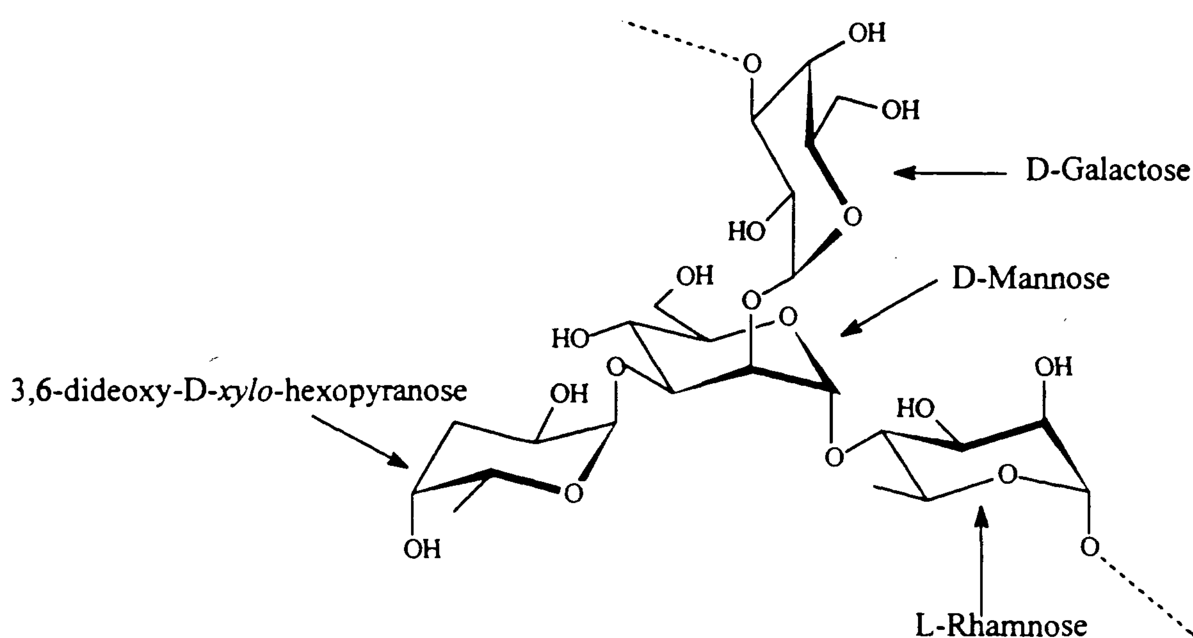


Figure 2.3. The branched tetrasaccharide repeating unit of the *O*-antigen of *Salmonella* serogroup B.

The carbohydrate binding characteristics of Se155.4 have been well studied. Se155.4 binds a trisaccharide portion, methyl 3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-2-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (**10**), of the *Salmonella* serogroup B *O*-antigen (88, 95). Detailed epitope mapping using modified and monodeoxy oligosaccharide derivatives of the *O*-antigen demonstrated that three

specific Ab-saccharide hydrogen bonds as well as complementary surfaces were involved in the interaction between Se155.4 and trisaccharide **10**. The three hydrogen bonds were contributed by the two hydroxyl groups of the 3,6-dideoxy-*xylo*-hexopyranose unit and from the 4-OH of the mannose residue. A bound water molecule was found at the bottom of the Ab binding pocket and was involved in a hydrogen bond network with the two hydroxyl groups of the 3,6-dideoxy-*xylo*-hexopyranose, a tryptophan from the light chain and a histidine from the heavy chain of Se155.4. When galactose was replaced with a methyl or methoxymethyl group, the resulting disaccharides were active, suggesting that galactose has only a minor role in Se155.4 recognition. Data involving other derivatives of the galactose residue suggest that the 4-OH and 6-OH are mainly involved in van der Waals interactions. An interesting interaction involving the galactose residue is the intersaccharide hydrogen bond between the 2-OH of galactose and the 2-OH of 3,6-dideoxy-*xylo*-hexopyranose (Figure 2.4). This hydrogen bond is only possible due to a conformational shift about the galactose-mannose linkage which occurs upon binding. The trisaccharide **10** presents a nonpolar surface to the Ab since all the hydroxyl groups involved in hydrogen bonds are found towards one face. Se155.4 provides a hydrophobic environment for the nonpolar face of the trisaccharide **10** by stacking a tryptophan from the light chain against the 3-C, 4-C, 5-C, and 6-C of the 3,6-dideoxy-*xylo*-hexopyranose unit and the 1-C and 2-C of galactose. A tryptophan from the heavy chain is stacked against the 6-C of the 3,6-dideoxy-*xylo*-hexopyranose unit and the 3-C of mannose. This aromatic stacking helps Se155.4 discriminate carbohydrates containing 3,6-dideoxy-glucose and the 4-C epimer of 3,6-dideoxy-*xylo*-hexopyranose. The thermodynamic

parameters of the interaction between trisaccharide **10** and Se155.4 were studied by titration microcalorimetry and it was suggested that the displacement of water from the mAb binding site accounted for the favorable entropy observed upon Se155.4 binding of the native trisaccharide (88).

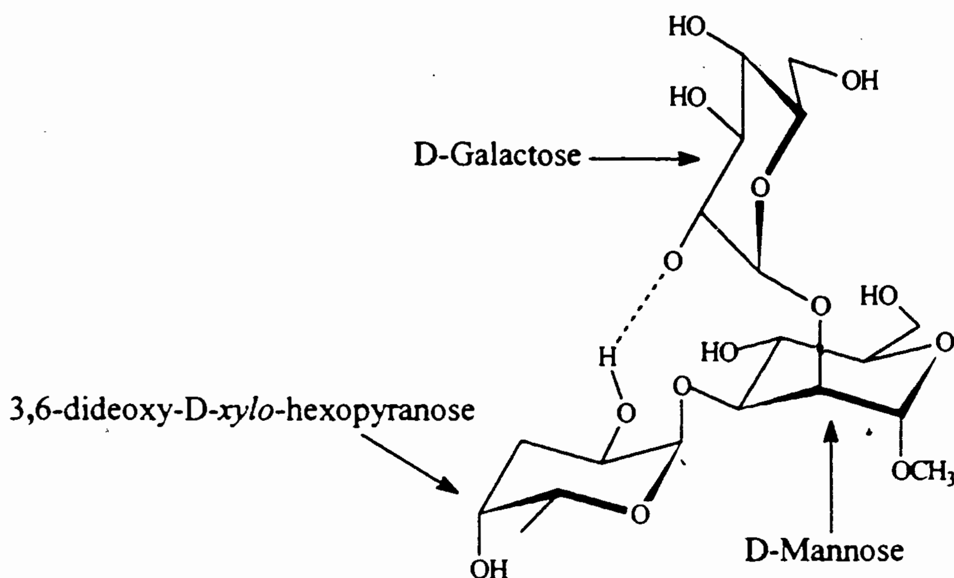


Figure 2.4. Schematic representation of the intersaccharide hydrogen-bond present in the trisaccharide **10** bound by Se155.4.

The dynamic and structural behavior of Se155.4 complexed with carbohydrate haptens were investigated by TRNOE experiments and analysis of crystal structures, respectively (88, 95, 96). The distance constraints derived from the TRNOE buildup curves suggested that two conformations of the trisaccharide **10** were bound by Se155.4. Moreover, TRNOE experiments demonstrated a protein-induced conformation shift about the galactose-mannose linkage of the trisaccharide **10**. This bound conformation correlates with the conformation observed in the 2.1 Å resolution crystal structure of Se155.4 complexed with the trisaccharide hapten **10** (88). This same conformation was

also observed in the crystal structure of the dodecasaccharide-Fab complex (96). The bound conformation has a shift about the galactose-mannose linkage that results in a short interatomic distance between the 2-OH of the 3,6-dideoxy-*xylo*-hexopyranose unit and the 2-OH of galactose, thus allowing the formation of an intramolecular hydrogen bond. As was the case with the Bra Tri (**4**) of GAS, this bound conformation of the *Salmonella* trisaccharide **10** is not the predominant conformation observed for the free ligand in solution.

2.4 The anti-*Shigella flexneri* Y mAb SYA/J6

The IgG3 mAb, SYA/J6, was raised against *Shigella flexneri* Y and is directed against the *O*-antigen of the LPS, which consists of a tetrasaccharide repeating unit, 2-acetamido-2-deoxy-3-*O*-((3-*O*-(2-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-β-D-glucopyranose (**ABCD**), shown in Figure 2.5 (97-99).

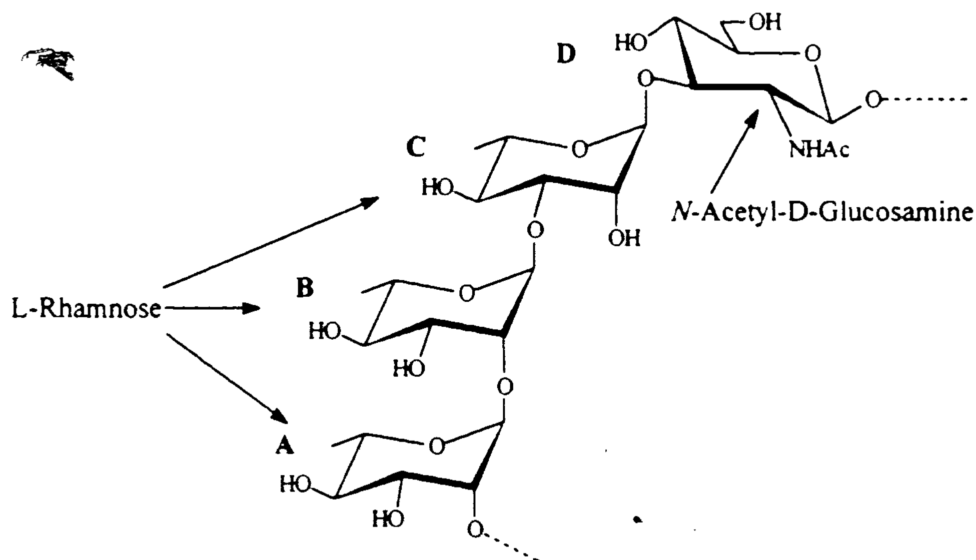


Figure 2.5. The tetrasaccharide repeating unit of the O-antigen of *Shigella flexneri* Y.

The sequence of SYA/J6, shown in Figures 2.2a and 2.2b, has been determined and is similar to the anti-dGAS mAbs. However, the gene usage cannot be assigned unambiguously and therefore cannot be compared to the gene usage of the anti-dGAS mAbs. Nevertheless, SYA/J6 does not bind dGAS (Table 2.4).

SYA/J6 has been shown to bind the tetrasaccharide methyl 2-acetamido-2-deoxy-3-*O*-((3-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**BCDA**, see Figure 2.5) (97-99). Data from inhibition ELISAs using chemically modified carbohydrate derivatives suggest that the C-6 of the Rha residue **C** contacts SYA/J6 while the C-6 of the Rha residue **B** and the acetamido of Glc*N*Ac are solvent exposed. The 4-OH of Glc*N*Ac has also been demonstrated to be a crucial polar contact between the carbohydrate and SYA/J6. It has been suggested that important van der Waals contacts are made between SYA/J6 and an extended non-polar surface on the carbohydrate that begins next to the 4-OH of the Glc*N*Ac and stretches from the α-face of the Glc*N*Ac to include the β-face of the Rha residue **C**.

The monodeoxy trisaccharide (**BC'D**), corresponding with a deoxy functionality at the 2-C of the **C**-ring, has been shown to have a higher affinity for SYA/J6 than the native tetrasaccharide (**ABCD**). Thus, the **BC'D** trisaccharide and a pentasaccharide **ABCDA** were complexed to SYA/J6 and their crystal structures were solved at 2.5 Å resolution (100).

2.5 Materials and Methods

The anti-dGAS CWPS mAbs, SA-2 (3), SA-3 (3), SA-4 (3), Strep 9 (5); (a generous gift from J. B. Pitner, Becton Dickinson Research Center, Durham, NC), HGAC 39.G3, HGAC 47.G3, HGAC 85.G3 and HGAC 101.G3 (4); (kindly provided by N. S. Greenspan, Case-Western University School of Medicine, Cleveland, OH), were raised against a heat-killed, pepsin-treated GAS (dGAS) vaccine (85). The dGAS used in this work was kindly provided by J. B. Pitner and D. R. Bundle (University of Alberta, Edmonton). The production and characterization of the pcAbs were described previously (3). The mAbs Se155.4 and SYA/J6 (kindly provided by D. R. Bundle) were raised against *Salmonella* serogroup B and *Shigella flexneri* Y, respectively. The amino acid sequences are published for mAbs Se155.4 (88), HGAC 39, HGAC 47 and HGAC 101 (4); those of Strep 9, SA-3, and SYA/J6 are published in reference 29. The syntheses of the GAS oligosaccharides and glycoconjugates have been published (3, 36, 89, 90).

2.5.1 Purification of SA mAbs by ammonium sulfate precipitation.

The ascitic fluid (3 mL) was cleared by centrifugation and the supernatant was passed through a 0.45 μm syringe filter, followed by a 0.22 μm syringe filter. The cleared ascitic fluid was mixed with an equal volume of ammonium sulfate (3 mL, saturated, pH 7) and allowed to stir at 4°C, overnight. The resulting precipitate was collected by centrifugation (10 000 x g, 30 min at 4°C) and resuspended in half the original volume of PBS (1.5 mL). After dialyzing overnight against PBS, the dialyzate was cleared by centrifugation.

2.5.2 Purification of pcAbs.

The sera from one group of rabbits was cleared by centrifugation and the supernatant was passed through a 0.45 μm syringe filter. A protein A-Sepharose (Pharmacia Canada Inc., Dorval, PQ) column with a small cup on top containing G-25 resin (Pharmacia Canada Inc., Dorval, PQ) was equilibrated with a binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9). The cleared supernatant was mixed in a 1:1 ratio with the binding buffer and loaded onto the column system. Unbound proteins were washed from the column with the binding buffer. Bound IgGs were eluted from the column using an eluting buffer (0.1 M citric acid, pH 3). One mL fractions were collected and the A_{280} was monitored. Fractions containing protein were immediately combined and dialyzed for 24 h at 4°C vs. a Tris buffer (0.1 M Tris/HCl, 0.5 M NaCl, pH 8). The dialyzate was concentrated using an Amicon ultrafiltration apparatus.

The protein A-Sepharose column was regenerated by washing with a regeneration buffer (0.1 M citric acid, pH 3) and was then washed in PBS (pH 7) and stored.

The anti-BSA pcAbs were subtracted from the total IgG fraction through the use of a BSA-Sepharose column (preparation described below). The IgG fraction was cleared by centrifugation and the supernatant was passed through a 0.8 μm syringe filter. The IgG fraction was loaded onto the BSA-Sepharose column which was equilibrated with a binding buffer (0.1 M Tris/HCl, 0.5 M NaCl, pH 8.5). The unbound IgGs, including those which bound the carbohydrate portion of the glycoconjugates used for the production of the pcAbs, were washed off the column using the binding buffer. Fractions

containing protein, as determined by the A_{280}, were combined and dialyzed vs. a Tris buffer (50 mM Tris/HCl, 150 mM NaCl, pH 8) overnight at 4°C.

The BSA-Sepharose column was regenerated by removing the bound anti-BSA pcAbs and washing the column with a low pH buffer (0.05 M glycine/HCl, 0.15 M NaCl, pH 2). Once all the bound protein was removed, the column was re-equilibrated with the binding buffer.

2.5.3 Preparation of BSA-Sepharose.

Cyanogen bromide-activated Sepharose 4B (15 g, Pharmacia Canada inc., Dorval, PQ) was suspended in 1 M HCl in order to swell the gel (1 g of dry gel swells to 3.5 mL). The swollen gel was washed with 1 M HCl (200 mL/ g of dry gel) using a sintered glass filter in order to remove additives with which the gel was freeze dried. The washed and swollen gel was suspended in a bicarbonate buffer (60 mL, 0.2 M $NaHCO_3/Na_2CO_3$, pH 9.5). A solution of BSA (241 mg in 5.0 mL of 0.1 M $NaHCO_3/Na_2CO_3$, pH 9.5) was added and the mixture was left at room temperature for 2.5 h with occasional stirring. The BSA-Sepharose was filtered on a sintered glass funnel and the A_{280} of the filtrate was measured in order to determine the percent incorporation of BSA.

In order to block any unbound sites on the Sepharose, the BSA-Sepharose gel was resuspended in a glycine-containing buffer (100 mL 0.2 M $NaHCO_3/Na_2CO_3$, 0.2 M glycine, pH 9.5) overnight at 4°C. The blocked BSA-Sepharose gel was filtered on a sintered glass funnel and washed with three cycles of buffer with alternating low and high pH. Each cycle consisted of a wash with a NaOAc buffer (200 mL, 0.1 M NaOAc, 0.5 M

NaCl, pH 4) followed by a wash with a Tris buffer (200 mL, 0.1 M TrisHCl, 0.5 M NaCl, pH 8.2). The washed and blocked BSA-Sepharose gel was stored at 4-8°C.

2.5.4 Competitive ELISA for carbohydrate epitope mapping

Preliminary set-up for inhibition assays vs. immobilized dGAS. The dGAS was diluted in carbonate buffer (0.05 M, pH 9.8) to concentrations of 10^9 organisms/mL, 10^8 organisms/mL, and 10^7 organisms/mL, added to a microtitre plate (40 μL/well) and left to bind overnight at 4 °C in a humid box. All plates were blocked with blotto (5% milk powder in Tris-buffered saline (TBS, 50 mM TrisHCL, 150 nM NaCl, pH 7.5, 200 μL/well) for 2h at room temperature and then washed three times with TBS.

Serial dilutions of mAb ranging from 100 μg/mL to 0.1 μg/mL (dilution factor of $\sqrt{10}$) were prepared in blotto. The mAb solutions were added to the plates (35 μL/well) and incubated for 2 h at 37 °C. Plates were then washed three times with TBS.

Biotinylated mAbs were detected with avidin:horseradish-peroxidase complexes, and non-biotinylated mAbs were detected with secondary Abs conjugated to horseradish peroxidase (Pierce, Rockford, IL). Optical densities are reported as (A_{405}-A_{490}) x 1000. The combination of immobilized dGAS and mAb concentration that produced an absorbance of approximately 0.7-0.8 was chosen for the inhibition ELISA protocol.

Inhibition Assays. Wells were coated with the concentration of dGAS determined from the preliminary set-up. Wells used to determine background values were coated

with blotto. After washing three times with TBS, the plates were blocked with blotto (200 µL/well) for 2h at room temperature and then washed three times with TBS.

The inhibitor dilutions were made at twice the final concentration in blotto. The final concentration range covered by the inhibitors was 0.0316 µg/mL to 1000 µg/mL (dilution factor of $\sqrt{10}$). The mAb solutions were prepared in blotto at twice the final concentration determined in the preliminary set-up. Equal volumes of inhibitor and mAb solution (20 µL each) were added to the microtitre plate. Control wells received blotto. The plates were incubated overnight at room temperature and then washed three times with TBS.

Biotinylated mAbs were detected with avidin:horseradish-peroxidase complexes, and non-biotinylated mAbs were detected with secondary Abs conjugated to horseradish peroxidase (Pierce, Rockford, IL). Optical densities are reported as $(A_{405}-A_{490}) \times 1000$.

CHAPTER 3

EVIDENCE FOR DISCRIMINATION BY PEPTIDES BETWEEN CLOSELY RELATED ANTI-CARBOHYDRATE ANTIBODIES

3.1 Introduction

Proteins (or peptides) have been shown to cross-react with carbohydrates, that is proteins have been shown to bind carbohydrate-reactive molecules. The first examples involved antibodies (Abs) directed against the variable domains of anti-carbohydrate Abs (13-19). These Abs are known as anti-idiotypic (Id) Abs. When some of these carbohydrate-cross-reactive anti-Id Abs were used themselves as immunogens, they elicited a new immune response that included Abs capable of binding the original carbohydrate antigen. This ability to elicit carbohydrate-binding Ab responses has been attributed to chemical similarity between an anti-Id Ab and the corresponding carbohydrate antigen (19); the anti-Id Ab is said to contain an internal image of the original antigen. More recently, peptide libraries have been used to identify cross-reactive peptides for several, anti-carbohydrate monoclonal antibodies (mAbs) (20-22). Except for the work by Valadon *et al.* (22), these reports of peptide cross-reaction with carbohydrate involved only single mAbs. Yet, Valadon *et al.* (22) used only one mAb to screen the phage-displayed peptide libraries. Two groups have attempted to use their cross-reactive peptides to elicit a carbohydrate reactive immune response. Valadon *et al.* (22) demonstrated that the peptide was able to elicit Abs having the same idiotype as the cognate, anti-carbohydrate mAb; Westerink *et al.* (23) used a cross-reactive peptide,

whose sequence was based upon a portion of an anti-Id Ab, to elicit a carbohydrate-binding response. In spite of these reports, the generality of discovering peptides capable of binding anti-carbohydrate mAbs and the mechanism of Ab-mediated cross-reaction between peptides and carbohydrates remained to be established.

This chapter investigates the molecular basis of the Ab-mediated cross-reactivity between proteins and carbohydrates. A panel of five closely-related mAbs directed against the CWPS of GAS, three related pcAbs directed against the CWPS of GAS and two mAbs directed against unrelated carbohydrate structures were used to isolate cross-reactive peptides from phage-displayed peptide libraries. Consequently, the isolation of cross-reactive peptides has been demonstrated for a large number of different anti-carbohydrate Abs.

The panel of closely related mAbs provided insight into the mechanisms involved in Ab-mediated peptide-carbohydrate cross-reactivity. All of the mAbs used for this work isolated cross-reactive peptides from a panel of eleven phage-displayed libraries, and a unique consensus sequence could be derived from the peptides isolated by most of the mAbs. Each consensus sequence group was chemically-distinct, as the peptide sequences were different between groups but very similar within groups. Moreover, representative peptides from each group were functionally specific, since they usually bound only to their isolating mAb. Restricted peptide specificity that did not follow carbohydrate reactivity was also observed for three polyclonal Abs directed against synthetic oligosaccharide fragments of the GAS CWPS. Thus, the predominating basis

of peptide recognition by anti-carbohydrate Abs differs between Abs, with true, carbohydrate mimics being relatively rare.

3.2 Results

3.2.1 SA-3 isolation of a carbohydrate-cross-reactive peptide.

SA-3, an anti-dGAS mAb, isolates a carbohydrate-cross-reactive peptide that is mAb specific. Lisa Craig used SA-3 to screen a 6-mer and a 15-mer library displayed on cpIII (Table 3.1). The data in Table 3.2 show that the phage clones isolated from the 6-mer library bear peptides sharing the consensus sequence **RPX_2Y**. In order to investigate whether SA-3 would recognize a peptide outside the context of the phage, a moderate-binding sequence (DRPVPY) which contained the consensus group and hydrophilic amino acids was synthesized. Since the peptides were displayed as fusions near the *N*-terminus of cpIII, the *N*-terminal amino acids of cpIII were included in the synthetic peptide; the sequence synthesized was NH_2ADGADRPVPYGACGOrn (biotin)-NH_2 (DRPVPY-peptide), where Orn represents ornithine, an unnatural amino acid. Orn was included to characterize any protein conjugates made with this peptide. The amount of peptide coupled to a naturally occurring protein could be determined by measuring the amount of Orn present in a sample of the synthetic conjugate since Orn would not be found in the protein carrier. The data in Table 3.3, obtained from competition ELISA, demonstrate that the DRPVPY-peptide inhibited SA-3 from binding any of the carbohydrate antigens, including dGAS. These data suggest that the DRPVPY-peptide binds at or near the carbohydrate-binding site. Moreover, the structure of the peptide is

not greatly influenced by the large phage coat protein since SA-3 recognized the isolated peptide.

Table 3.1. The phage-displayed peptide libraries

Library Structure	Description[a]
X_6[b]	cpIII-displayed linear sequence 6 amino acids long
X_{15}	cpIII-displayed linear sequence 15 amino acids long
X_6	linear sequence 6 amino acids long
X_{15}	linear sequence 15 amino acids long
X_{30}	linear sequence 30 amino acids long
XCX_4CX	a 4-mer loop constrained by a fixed disulfide bridging
XCX_6CX	a 6-mer loop constrained by a fixed disulfide bridging
XCX_8CX	a 8-mer loop constrained by a fixed disulfide bridging
X_8CX_8	one fixed cysteine residue flanked by 8-mer sequences that may contain cysteine in random positions, thus this library has the potential to form disulfide bridged loops of varying sizes
$X_{15}CX$	one fixed cysteine residue flanked by a 15-mer sequences that may contain cysteine in random positions, thus this library has the potential to form disulfide bridged loops of varying sizes
XCX_{15}	one fixed cysteine residue flanked by a 15-mer sequences that may contain cysteine in random positions, thus this library has the potential to form disulfide bridged loops of varying sizes
$X_4CX_4CX_4$	a 4-mer loop constrained by disulfide bridging and flanked by 4-mer linear sequences
$X_4CX_6CX_4$	a 6-mer loop constrained by disulfide bridging and flanked by 4-mer linear sequences
$XCCX_3CX_5C$	a library based upon the structure of alpha conotoxin

a) all libraries are displayed on cpVIII unless otherwise noted

b) X represents randomized amino acids and C represents fixed cysteines

Table 3.2. The phage-displayed peptide sequences isolated by the anti-dGAS mAbs, Se155.4 and SYA/J6

mAb	Library	Peptide Sequence	ΔOD x1000
SA-2	X_6[a,b]	RVCFVC	n/d[c]
		KCCYSV	n/d
		DCVFLS	n/d
SA-3	X_6[a]	**DRPVPY**[d,e]	**765**
		KSPTPY	641
		ARPLWY	583
		VRPQVP	524
	fd-tet[f]	---	169
SA-4	X_6[a]	WFDPDF	n/d
		WYDPDF	n/d
Strep 9	XCX_8CX[g]	**MCPPLYSPSACA**	**957**
		ECNFLYPGFTCA	202
	X_8CX_8[g]	**YPYCGHALCPGLYADAS**	**1020**
		VILPYDNNCALCLNLYP	644
		VIDAPTPNCAWPNGRRG	256
		MPPAGTGTCFLYALSCS	153
		ADLSPTPYCQPSTMHTN	144
		NEYINQDHCLLYAMLCP	38
	$X_{15}CX$[g]	EIAPQGPSKCLLYAYCQ	13
	f88[f]	---	14
HGAC 39	X_{15}[g]	**ADAAPSPTPYLPRLS**	**643**
		ATYRPVPAEFARKHL	425
		TITATDSPTPWPFER	244
	XCX_8CX[g]	MCRPSPYNPPCT	112
	f88	---	72
HGAC 47	XCX_8CX[g]	**MCRPGIPTHHCA**	**174**
		HCSPGQRPGTCQ	165
		DCGNMLHAEVCR	**149**
		DCRPGVPLLSCP	115
	f88	---	87
HGAC 101	XCX_6CX[g]	**SCISAACFCI**	**141**
	X_6[g]	**KQLMAP**	**138**
	f88	---	29

Table 3.2 (concl'd). The phage-displayed peptide sequences isolated by the anti-dGAS mAbs, Se155.4 and SYA/J6

mAb	Library	Peptide Sequence	ΔOD x1000
Se155.4	X_6[g]	**NYPMDH**	**141**
		MYPMSH	104
		YPMGHL	27
		IYPMPA	13
		QQYPMG	12
		QSTYPM	10
	X_{15}[g]	**EPYPMSEANYVRPMP**	**267**
		YPMPASSDNAQWLLK	15
		DGTNAYPMNEDISVS	15
		HSTRNYSYLGSPYPM	13
		NYPMSGARIEPLLHA	13
		YAATEPRYMIPYPMP	13
	XCX_8CX[g]	VCPAPYPAGTCA	11
	f88	---	14
SYA/J6	$X_4CX_4CX_4$[g]	**YTTQCGYGGCMNFE**	**938**
		MGVICMNMECDRNM	**799**
		LHEYCNMETCPYNH	585
		QYPQCHNMDCKSIT	472
		PTHVCYNMECQGGD	275
		TPTNCYNMTCQNQP	187
	X_6[g]	**MDWNMH**	**993**
	f88	---	66

a) the listed peptides displayed on cpIII are preceded by the *N*-terminal sequence ADGA
b) sequence of the peptide library, in which X represents "randomized" amino acids and C stands for fixed cysteines
c) not determined
d) sequences in bold were chosen for further study; see Table 3.5
e) consensus sequences are underlined
f) wild-type phage vector without a random peptide insert
g) the listed peptides are displayed on cpVIII

Table 3.3. Percent inhibition by the DRPVPY-peptide, Penta (7) or dGAS of SA-3 binding to immobilized antigens

	Inhibitor in solution		
Immobilized antigen	DRPVPY-peptide (500 nM)	Penta (7) (30 μM)	dGAS (3.5 x 10^6)[a]
Di (2)-BSA (10^{11})[b]	88%	63%	64%
Lin Tri (3)-BSA (10^{11})[b]	88%	62%	42%
Bra Tri (4)-BSA (10^{11})[b]	88%	80%	57%
Penta (7)-BSA (10^{11})[b]	15%	2%	4%
CWPS-BSA (10^{14})[b]	27%	5%	20%
dGAS (4 x 10^7)[a]	22%	17%	n/d[c]
DRPVPY-peptide (10^{11})[b]	40%	50%	24%
DRPVPY phage[d](4 x 10^{14})[b]	84%	58%	51%

a) number of bacterial cells added to microwell
b) number of hapten molecules added to each microwell
c) not determined
d) phage clone displaying the peptide sequence DRPVPY on cpIII

The data in Table 3.3 suggest that the affinity of SA-3 for the DRPVPY-peptide is greater than for any of the oligosaccharides since a lower concentration of the DRPVPY-peptide is needed for inhibition of SA-3 binding to immobilized carbohydrate antigens. The binding of SA-3 to the DRPVPY-peptide and to Hexa 2 (**9**), one of the best binding oligosaccharides, was further investigated by titration microcalorimetry. The titration microcalorimetry was performed by K. Kenar and E. J. Toone at Duke University. The titration curves shown in Figure 3.1 were well behaved. As shown in Table 3.4, the K_a of SA-3 for the DRPVPY-peptide was 30 times greater than for Hexa 2 (**9**), an oligosaccharide whose inhibitory potency was similar to that of purified CWPS. The enthalpies of binding, shown in Table 3.4, for the DRPVPY-peptide and Hexa 2 (**9**) were similar; thus, the difference in affinity was due to the calculated entropies of binding.

Deconvolution of the data obtained from the microcalorimetry experiments provides a least-squares estimate of Ka, the enthalpy of binding, ΔH_0, and the number of binding sites, n (121). By assuming 1:1 stoichiometry for the interaction of the ligand with the mAb the experimental data can be fit by the equation

$$1/V_0(dq/dL_{tot}) = \Delta H_0 \left[1/2 + \frac{1 - (1+r)/2 - L_r/2}{(L_r^2 - 2L_r(1-r) + (1+r)^2)^{1/2}} \right]$$

where V_0 = reaction cell volume

q = heat absorbed or evolved

L_{tot} = total amount of ligand

$1/r$ = {(total amount of protein) x K}

$L_r = L_{tot}$/(total amount of protein)

Thus ideally, n should equal 1. As can been seen in Table 3.4, while n was not 1, indicating that the SA-3 sample was contaminated with irrelevant protein, it was determined to be the same for both the DRPVPY-peptide and Hexa 2 (9). Finding the same stoichiometry of interaction provides support, independent from the competition ELISAs, for peptide binding in the carbohydrate-binding site of SA-3.

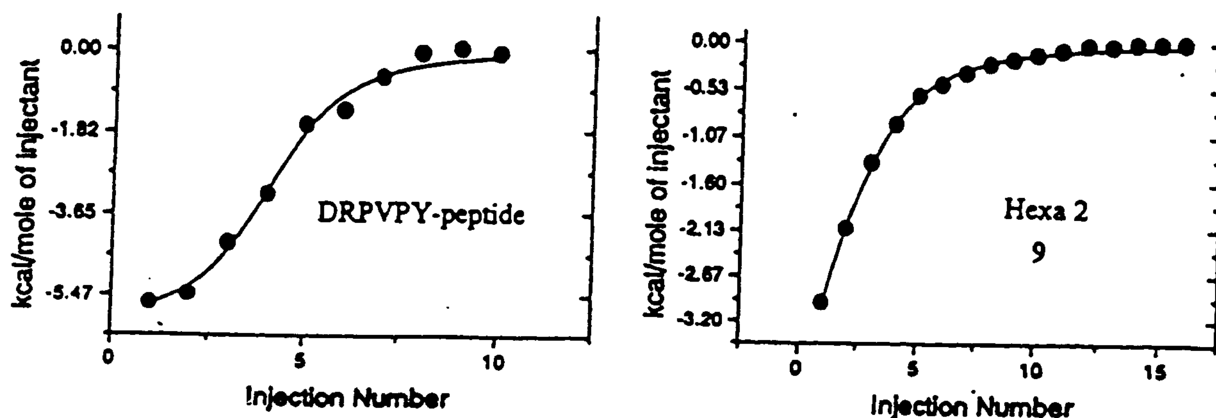


Figure 3.1. The titration curves for SA-3 binding to Hexa 2 and to DRPVPY-peptide.

Table 3.4. Thermodynamic parameters of SA-3 binding to DRPVPY-peptide and Hexa 2 (9)

	DRPVPY-peptide	Hexa 2 (9)
K_a (M^{-1})	$(1.6 \pm 0.4) \times 10^6$	$(5.7 \pm 0.5) \times 10^4$
ΔG (kcal/mol)	$-(8.46 \pm 0.35)$	$-(6.49 \pm 0.05)$
ΔH (kcal/mol)	$-(6.30 \pm 0.33)$	$-(5.62 \pm 0.38)$
TΔS (kcal/mol	2.16 ± 0.68	0.87 ± 0.43
n	0.376 ± 0.015	0.398 ± 0.023

The data in Table 3.5 demonstrate that, besides SA-3, none of the anti-dGAS mAbs bound the DRPVPY-peptide. The lack of peptide binding to HGAC 39 is particularly intriguing, given its similarities to SA-3 in sequence and oligosaccharide-binding properties (Figures 2.2a and 2.2b, Table 2.1). In order to determine if other anti-dGAS mAbs were capable of binding peptides, we extended our study to the other mAbs in the panel.

Table 3.5. Binding of clones isolated by an anti-carbohydrate mAb with other anti-carbohydrate mAbs

	mAb						
Immobilized antigens	SA-3	Strep 9	HGAC 39	HGAC 47	HGAC 101	SE 155.4	SYA/J6
DRPVPY-peptide	**300**[a]	28	21	20	24	n/d	n/d
BSA[b]	52	22	16	15	17	n/d	n/d
MCPPLYSPSACA[c]	92[d]	**446**	40	44	25	10	33
YPYCGHALCPGLYADAS	7	**674**	42	45	22	8	29
ADAAPSPTPYLPRLS	47	14	**726**	62	31	12	35
MCRPGIPTHHCA	6	13	44	**148**	29	11	33
DCGNMRQAEVCR	7	12	41	**160**	26	10	32
SCISAACFCI	6	12	80	120	**51**	12	36
KQLMAP	6	12	95	121	**43**	11	34
NYPMDH	6	12	58	78	37	**112**	36
EPYPMSEANYVRPMP	6	11	56	74	32	**145**	35
MDWNMH	6	16	57	69	34	13	**689**
MGVICMNMECDRNM	6	12	47	54	28	10	**283**
YTTQCGYGGCMNFE	8	14	55	55	32	12	**483**
f88[e]	7	11	48	55	26	10	34

a) numbers in bold indicate the clone was detected by the corresponding mAb (e. g., SA-3 isolated the clone bearing the DRPVPY sequence).

b) BSA-coated wells used as a negative control for DRPVPY-peptide-coated wells

c) underlined sequences indicate unique consensus groups

d) underlined numbers indicate significant binding by a mAb not used to select the clone

e) the wild-type phage vector without a random peptide insert

3.2.2 All of the closely related, anti-dGAS mAbs isolate peptides having restricted specificity.

Jarnail Mehroke and Michael Zwick used Strep 9, HGAC 39, HGAC 47, HGAC 85 and HGAC101, other closely-related anti-dGAS mAbs, in four rounds of biopanning with a panel of peptide libraries displayed on cpVIII (30). The random peptide sequences in each library varied in length, and in the number and position of fixed cysteine residues within the randomized region (Table 3.1) (30).

In order to conserve space during the biopanning experiments, the HGAC mAbs were mixed together in pairs: HGAC 39 with HGAC 85 and HGAC 47 with HGAC 101. After four rounds of biopanning, each HGAC mAb was tested separately, by ELISA, for its ability to bind the enriched phage pools. HGAC 85 bound f88.4, phage with no peptide insert, with an optical density of 0.1, approximately twice that of the other HGAC mAbs (see Table 3.5); therefore, this mAb was not investigated further. The enriched pools of phage from only three of the phage-displayed libraries tested were selected for further biopanning. The libraries that would provide complementary structural information and that had large ELISA signals were chosen. The phage pools from the third round of biopanning were used again for another round of biopanning; however, for this fourth round of biopanning, the mAbs were used separately. The two pools from this final biopanning which had the largest ELISA signals were chosen for further study.

The data in Table 3.2 illustrate that each mAb isolated binding clones, and that Strep 9, and HGAC 39 (and SA-3) isolated tighter-binding clones than did HGAC 47 or HGAC 101, paralleling their reactivities with the oligosaccharides (Table 2.1).

Sequencing experiments performed by Michael Rashed on the oligonucleotide inserts of isolated phage clones demonstrated that each of the anti-dGAS mAbs, other than HGAC 101, isolated unique consensus groups (underlined in Table 3.2); these are **$CX_{1-2}LY$** and **PTPXC** for Strep 9, **$PXPX_{1-2}P$** for HGAC 39, and **CXPG** or **RPG** for HGAC 47. Thus, the mAbs that bound the GAS CWPS epitope tightly isolated tight-binding peptides which formed consensus sequences. In contrast, the weak-binding mAbs bound peptides relatively weakly, and either isolated a weak consensus sequence or none at all. Two other anti-dGAS mAbs, SA-2 and SA-4 (3), also isolated binding peptides having unique consensus sequences (Table 3.2). However, since the carbohydrate-binding characteristics of these two mAbs could not be determined, these results were not investigated further.

Further examination of the sequences in Table 3.2 reveals that two common motifs were identified by several of the mAbs. The sequence $ADX_{(1-3)}SPTPY$ was present among the clones isolated by SA-3 (ADGAKSPTPY), Strep 9 (ADLSPTPY...) and HGAC 39 (ADAAPSPTPY...), the mAbs that bound the GAS CWPS tightly. A second motif $RPX_{(1-2)}P$ was isolated by SA-3 (...RPVP..., ...RPQVP...), HGAC 39 (...RPVP..., ...RPSP...), HGAC 47 (...RPGIP..., ...RPGVP...) and Se155.4 (...RPMP). As discussed below, the functional relevance of these motifs is not obvious, since peptides sharing these motifs do not cross-react with each other.

The observations of distinct consensus groups and shared sequence motifs between groups led us to question the specificity of the peptides for each mAb. To test this, we determined the reactivity of each mAb with the best-binding phage pools isolated

by each mAb used in this study. In some cases, more than one mAb bound detectably to a given phage pool; however, none of the pools bound all of the mAbs, or even the complete group of mAbs that bound the GAS CWPS tightly (Table 3.6). To confirm these results, each mAb was tested for binding to the best-binding clones from each pool by ELISA; their peptide sequences are shown in bold in Table 3.2. The data in Table 3.5 show that each clone was bound best by the mAb that isolated it, with the exception of the clones isolated by HGAC 101. Importantly, where reactivity of a clone with more than one anti-dGAS mAb occurred (excluding the clones isolated by HGAC 101), it was limited to only a single mAb. This shows that none of the peptides follow the broad reactivity patterns observed with oligosaccharides like the Bra Tri (**4**) (Table 2.1). The reactivity patterns paralleled those of the phage pools from which the clones were derived, indicating that the clones tested represent the predominating reactivities in the phage pools.

Table 3.6. Binding of enriched phage pools isolated by an anti-dGAS mAb with other anti-dGAS mAbs

	mAb				
Library	SA-3	Strep 9	HGAC 39	HGAC 47	HGAC 101
X_6[a,b]	**305**[c]	21	35	62	46
XCX_8CX[d]	<u>551</u>[e]	**186**	34	43	44
X_8CX_8[d]	<u>72</u>	**79**	35	48	46
$X_{15}CX$[d]	15	**72**	37	44	45
X_{15}[d]	<u>588</u>	24	**294**	101	124
XCX_8CX[d]	<u>120</u>	23	**87**	61	71
XCX_8CX[d]	16	20	<u>105</u>	**324**	<u>160</u>
XCX_6CX[d]	12	21	<u>150</u>	<u>202</u>	**203**
X_6[d]	24	17	<u>156</u>	<u>219</u>	**215**
f88[f]	15	24	50	52	69

a) the listed peptides displayed on cpIII are preceded by the *N*-terminal sequence ADGA.
b) sequence of the peptide library, in which X represents a "randomized" amino acids and C stands for fixed cysteines
c) numbers in bold indicate the library was biopanned by the corresponding mAb (e. g., SA-3 was used to biopan the X_6 library).
d) the listed peptides are displayed on cpVIII.
e) underlined numbers indicate significant binding by a mAb not used to biopan the corresponding library.
f) the wild-type phage vector without a random peptide insert.

The best candidates for carbohydrate-mimic peptides are those that bind to multiple mAbs and/or bear the shared motifs found among multiple consensus groups. The best examples of peptides reacting with more than one mAb were for clones isolated by Strep 9 or HGAC 39 that were also bound by SA-3 (see underlined values in Table 3.5). The HGAC 39-isolated sequence ADAAPSPTPY... bears the $ADX_{1-3}SPTPY$ shared motif and bound very tightly to HGAC 39, yet only weakly to SA-3, and not detectably to Strep 9. The tight-binding, Strep 9-isolated sequence MCPPLYS... bound moderately-well to SA-3, but not detectably to HGAC 39; moreover, neither of the shared motifs are present in this peptide. None of the clones bearing the $RPX_{1-2}P$ motif, even the tight-

binding DRVPVY-peptide, bound detectably to mAbs other than the ones used for their isolation. Thus, no peptide was recognized by all of the anti-dGAS CWPS mAbs, or even the group that bound carbohydrate tightly, and shared motifs, although present, did not appear to be functional. It may be that the variability in the number of "X" residues separating the consensus residues in the common motifs (ADX$_{1-3}$SPTPY and RPX$_{1-2}$P) plays a role in conferring mAb-binding specificity to a given peptide.

The ability of Penta (7), one of the best-binding, synthetic oligosaccharides to compete with immobilized phage for mAb binding was tested by ELISA. The data in Table 3.7 show that a relatively high concentration of Penta (7, 1.1 mM) inhibited the binding of Strep 9 and HGAC 39 to their respective clones, indicating that peptide binding to these mAbs occurs at or near the carbohydrate-binding site. In contrast, this concentration of Penta (7) did not inhibit the binding of HGAC 47 or HGAC 101, suggesting that peptide binding occurs in a separate site. Since these latter mAbs appear to have a relatively lower affinity for carbohydrates than for peptides, it is also possible that inhibition may occur with a higher concentration of Penta (7).

Table 3.7. Percent inhibition by Penta (7) of anti-dGAS mAb binding to immobilized phage

mAb	Peptide displayed on phage	Percent inhibition by Penta (7, 1.1 mM)
Strep 9	MCPPLYSPSACA	94%
HGAC 39	ADAAPSPTPYLPRLS	67%
HGAC 47	HCSPGQRPGTCQ	0%
HGAC 47	DCGNMRQAEVCR	0%
HGAC 101	SCISRSSFCI	0%
HGAC 101	KQLMAP	0%

3.2.3 pcAbs directed against synthetic oligosaccharide fragments of the GAS CWPS also isolate cross-reactive peptides.

Our study was extended to pcAbs directed against BSA conjugates of the Lin Tri (**3**), Bra Tri (**4**), or Penta (**7**) (3). The specificities of the pcAbs were shown to be similar to those of the anti-dGAS mAbs, in that the anti-Lin Tri and anti-Bra Tri pcAbs bound only their cognate trisaccharide (**3, 4**) and Penta (**7**), whereas anti-Penta pcAb bound all three haptens (3); moreover, dGAS was bound best by the anti-Penta pcAb (Table 2.3). Lisa Craig used the pcAb to screen the cpIII phage-displayed peptide libraries shown in Table 3.1. These biopanning experiments yielded several unique consensus groups for each pcAb which are shown in Table 3.8. The different peptide sequences reflect the different Ab specificities within each pcAb. Hence, the existence of peptides capable of cross-reacting with carbohydrate has now not only been demonstrated for several mAbs, but three pcAbs as well. Since each pcAb is a collection of many different Abs, the isolation of cross-reactive peptides by this large collection of different anti-carbohydrate Abs strongly suggests that cross-reactive peptides can be found for most anti-carbohydrate Abs.

Table 3.8. The peptide sequences isolated by the pcAbs

pcAb	Peptide sequences[a]			
anti-Lin Tri pcAb	CVFHQD[b]	YLFTQD[c]	CVFHQD[b]	
	YLFTQD[c]	GYMFTQ	SKCNQP	
			KCSIRQ	
			LLACSY	
			LCQTCA	
anti-Bra Tri pcAb	SIKWLE	VSFWDW	AVWGPAGPAFRPRWS[d]	
	LIKWLE	FIFYPW	DWRFSFRPWGLDLSS	
	YWKYES			
	KFGDLF			
	AVWGPAGPAFRPRWS[d]		RDHLVFWTTSGPIFG	
	QMWFPAGPAWSSSCL		RDWHGAPYEVAVRSR	
anti-Penta pcAb	KCCVSV		YGYLYI	LVFYDD[e]
	LCCEGS		YSNLYL	DLLWDH
	CCSRFL		VNYSFY	FEFDYN[f]
	CCPTPC		YRNLLF	
	AVCCPCPSGSLPFFL			
	LFFAWY		WLLCVLVSDGFEFCAF	
	TRCLFFRGLSHCDVD		FEFDYN[f]	
	CDRQPPPVRCFRLVD			
	VPVWLATFRWEFYPF			
	LVFYDD[e]			
	WYWCYCIPLQLDDGC			

a) random peptide sequences displayed on cpIII are preceded by the N-terminal sequence ADGA

b-f) each superscript marks a sequence that appears twice because it aligns into two different consensus groups

The specificity of the phage pools obtained after three rounds of panning for their isolating pcAb was tested by ELISA. The data in Table 3.9 illustrate that each pcAb was specific for the phage pool it isolated, even though the pcAbs reacted with at least two of the three glycoconjugates used for the production of the pcAbs (3). Therefore, as with the

anti-dGAS mAbs, each pcAb was more specific for a given set of peptides than for the oligosaccharide antigens.

Table 3.9. Binding of pooled phage isolated by one pcAb with all pcAbs

	pcAbs		
Immobilized phage	anti-Lin Tri	anti-Bra Tri	anti-Penta
X_6[a]	**461**[b,c]	101	36
X_6	52	**222**	25
X_6	61	117	**262**
X_{15}	**286**	111	21
X_{15}	67	**299**	21
X_{15}	86	130	**90**
fd-tet[d]	80	158	37

a) X_6 and X_{15} indicate that phage pools were derived from the cpIII displayed 6-mer and 15-mer libraries, respectively
b) numbers are optical densities reported as $(A_{405}-A_{490}) \times 1000$
c) numbers in bold indicate that the listed phage pool was selected by the corresponding pcAb
d) wild-type phage clone without a random peptide insert

3.2.4 Two mAbs against non-GAS carbohydrates also isolate unique, cross-reactive peptides.

We extended our investigation to two well-characterized mAbs, Se155.4 and SYA/J6, which are specific for two different *O*-antigens. These "out-group" mAbs do not bind the GAS CWPS, even though the V_H-gene usage of SYA/J6 is similar to that of the anti-dGAS mAbs (Figures 2.2a and 2.2b). The work presented thus far has involved Abs directed against GAS carbohydrate structures only. Hence, the isolation of cross-reactive peptides by mAbs specific for different carbohydrate structures provides further evidence that cross-reactive peptides can be found for most anti-carbohydrate mAbs. Moreover, a variety of physical methods, including epitope mapping (88, 98, 99) and x-ray

crystallography (95, 96, 100), have been used to determine the features of the O-antigen of *Salmonella* serogroup B recognized by Se155.4, and of the *O*-antigen of *Shigella flexneri* Y recognized by SYA/J6.

Jarnail Mehroke and Michael Zwick used Se155.4 and SYA/J6 to biopan the same panel of cpVIII-displayed libraries screened by all the anti-dGAS mAbs, except SA-3 (Table 3.1). Sequencing experiments performed by Michael Rashed on the oligonucleotide inserts of isolated phage clones demonstrated that the peptide sequences isolated by Se155.4 share the very strong consensus sequence **YPM**, indicating strong selection by Se155.4 despite the low ELISA signals, whereas SYA/J6 isolated tight-binding peptides that form the unique consensus sequence **CXNM(E/D)** (Table 3.2). Two or three of the best-binding clones were tested for cross-reactivity with the other mAbs (Table 3.5); each was specific for its isolating mAb; nor did Se155.4 or SYA/J6 bind clones isolated by the other mAbs. In competition ELISA, the SYA/J6-specific trisaccharide methyl 2-acetamido-2-deoxy-3-*O*-((3-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside (98) at 1.8 mM inhibited the binding of SYA/J6 to phage; however, no detectable inhibition was observed for Se155.4 with 2.0 mM methyl 3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-2-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (95), a trisaccharide specific for this mAb (Table 3.10). These results suggest that peptide binding occurs at or near the carbohydrate-binding site of SYA/J6, but not of Se155.4.

Table 3.10. Percentage inhibition by trisaccharides of Se155.4 or SYA/J6 binding to immobilized phage

mAb	Peptide displayed on phage	Percent inhibition by a trisaccharide
Se155.4[a]	MYPMSH	0%
Se155.4	NYPMDH	0%
SYA/J6[b]	YTTQCGYGGCMNFE	74%
SYA/J6	PTHVCYNMECQGGD	100%

a) Se155.4 binding inhibited by 2mM methyl 3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-2-*O*-α-D-galactopyranosyl-α-D-mannopyranoside.

b) SYA/J6 binding inhibited by 1.8 mM methyl 2-acetamido-2-deoxy-3-*O*-((3-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside.

3.2.5 The requirement for disulfide bridging within the peptides isolated in this work.

mAb binding to those peptides shown in Table 3.5 containing two cysteines was determined in the presence and absence of 5 mM dithiothreitol. In every case, except SYA/J6, disruption of the disulfide bridging significantly decreased binding to the phage-borne peptides, but not to immobilized carbohydrate antigens (Table 3.11). Although disulfide bridging appears to be important for anti-dGAS mAb recognition, it is not always necessary, since each mAb also isolated linear peptides with relatively high affinity (Table 3.2). Cysteine residues, but not disulfide bridging, appear to be important for SYA/J6 recognition, since most of the tight-binding clones isolated came from the $X_4CX_4CX_4$ library, yet binding to these clones was unaffected by reducing conditions. Thus, in several cases, the constraints imposed by disulfide bridging provide a boost to affinity.

Table 3.11. Binding of mAbs to dithiothreitol (DTT)-treated clones

	Immobilized antigens					
	Isolated phage clone		f88[a]		Native antigen	
mAb	no DTT	with DTT	no DTT	with DTT	no DTT	with DTT
Strep 9[b]	752[c]	34	59	34	283	319
Strep 9[d]	901	34	35	33	283	319
HGAC 47[e]	147	46	60	33	158	148
HGAC 47[f]	177	44	60	32	162	162
HGAC 101[g]	166	74	55	27	154	110
SYA/J6[h]	836	788	26	30	762	760

a) the wild-type phage clone without a random peptide insert

b) binding to phage bearing the peptide MCPPLYSPSACA and to GAS, the native antigen

c) (A_{405}-A_{490}) x 1000 determined by direct ELISA

d) binding to phage bearing the peptide YPYCGHALCPGLYADAS and to dGAS, the native antigen

e) binding to phage bearing the peptide MCRPGIPTHHCA and to dGAS, the native antigen

f) binding to phage bearing the peptide DCGNMLHAEVCR and to dGAS, the native antigen

g) binding to phage bearing the peptide SCISAACFCI and to dGAS, the native antigen

h) binding to phage bearing the peptide YTTQCGYGGCMNFE and to *Shigella flexneri* Y LPS, the native antigen

3.3 Discussion

Our results with a panel of closely-related, anti-dGAS mAbs suggest that the mechanism of peptide binding often differs from that of carbohydrate binding. Most or all of the anti-dGAS mAbs probably bind carbohydrates by a similar mechanism, since they recognized the minimal, branched-trisaccharide epitope, and have similar V_H-gene usage. In contrast, each mAb recognized a restricted, mostly non-overlapping, subset of peptides. If peptide binding was solely due to mimicry of the GAS CWPS, the peptides should also have bound all of the anti-dGAS mAbs. Yet, cross-reactive peptides forming chemically-distinct consensus sequences were isolated for every anti-carbohydrate mAb

used in this work; moreover, the predominant reactivity of each peptide studied (besides those isolated by HGAC 101) was associated with the mAb that isolated it. The work with related pcAbs further supports this trend, as the phage pools isolated by each pcAb were not recognized by the other two pcAbs (Table 3.9), even though each pcAb recognized at least two of three glycoconjugates used for the generation of the pcAbs. We did find shared motifs between consensus groups that could potentially act as carbohydrate mimics; however, these peptides did not behave as carbohydrate mimics, as they reacted with no more than two of the anti-dGAS mAbs.

With regard to the definition of the carbohydrate epitope recognized by each anti-dGAS mAb, our oligosaccharide mapping studies (see Chapter 2) indicate that all of the anti-dGAS mAbs recognize an epitope presented by the branched-trisaccharide unit (**CBA'**). We infer from these data that the specific interactions (e.g., those of crucial hydroxyl groups involved in hydrogen-bonding networks between the mAb and the carbohydrate, or hydrophobic interactions between methyl groups on the carbohydrate and aromatic side chains) as well as the non-specific interactions (e.g., solvent displacement by sugar residues) are similar for the different anti-dGAS mAbs. It is possible, however, that the mAbs recognize different subsites within the branched-trisaccharide epitope and/or different conformations of the epitope, which would cause the crucial interactions between a mAb and the carbohydrate epitope to vary. Our study of the binding of the Bra Tri (**4**) to Strep 9 shows that this mAb selects a local minimum conformation that differs significantly from the global minimum conformation of the free trisaccharide in the ψ torsion angle about the Rha-α-(1→2)-Rha glycosidic linkage (92).

Thus, it is possible that the other anti-dGAS mAbs in the panel recognize the branched-trisaccharide epitope in alternative conformations and/or at other key subsites. It remains to be shown whether the other mAbs in the anti-dGAS panel select different conformations of the Bra Tri (**4**), which would indicate different mechanisms of binding; however, it would be surprising if each of the anti-dGAS mAbs were to bind a different conformation of the carbohydrate epitope.

The restricted reactivity of the peptides with the anti-dGAS mAbs shows that the peptides discriminate between the mAbs far better than the branched-trisaccharide epitope. This discrimination occurs even if some of the shared residues present in different consensus sequences are acting as structural mimics of the carbohydrate epitope; other residues must be responsible for discrimination. We conclude that the peptides bind the mAbs by different mechanisms than the branched-trisaccharide epitope. While the peptides present a surface that is complementary to the mAb combining site, the peptides must interact differently with the mAb combining sites in order to account for their increased discrimination. Alternatively, peptide binding may indeed be due to structural carbohydrate-mimicry; however, this would require the unlikely restriction that each mAb recognize the branched-trisaccharide epitope by a different mechanism (e.g., via different subsites).

The peptide specificity obtained with these anti-carbohydrate Abs has not been observed for other panels of related mAbs (unpublished data of L. Bonnycastle, L. Rees, E. Leong, K. Brown, J. Mehroke and J. Scott); these systems (mentioned in ref. 30) include (i) anti-peptide mAbs that bind the same residues on the peptide-immunogen, but

differ in their ability to bind influenza-virus hemagglutinin bearing the epitope sequence, (ii) anti-protein mAbs that bind the same discontinuous epitope on the gp120 of HIV-1, but differ in their ability to neutralize viral infectivity, and (iii) a mixture of (not necessarily closely-related) anti-peptide and anti-protein mAbs that have overlapping specificities for bacterial pilins. In the first two cases, the mAbs within each panel isolated the same or similar consensus sequence groups, and bound the peptides isolated by the other mAbs in the panel. In the third case, there was considerable variation in the sequences isolated by each mAb, but peptides from one of the consensus sequence groups were able to bind all of the Abs. These results are expected findings for panels of closely-related mAbs and highlight the extraordinarily-selective peptide reactivity of the anti-dGAS CWPS mAbs and anti-oligosaccharide pcAbs observed here.

Our work with seven anti-dGAS mAbs, three pcAbs, and two anti-lipopolysaccharide mAbs, when taken together with previous work (20-23), demonstrates that cross-reactive peptides can be found for most, if not all, anti-carbohydrate Abs. Peptides sharing a preference for **arXar** (where **ar** is an aromatic amino acid) have been previously reported to bind three different anti-carbohydrate mAbs (20, 22, 23), a lectin (24, 25) and one carbohydrate-reactive enzymes (73). This is probably not a general carbohydrate-mimic motif, since in our work, only a few peptides isolated from the pcAb screens had this motif; moreover, no other functional, shared motif was observed.

The peptides isolated by each mAb fell along a continuum of affinities, with the tightest-binders sharing a consensus sequence. Similar results have been reported by Valadon *et al.* (22), who used the neutralizing mAb 2H1 (raised against the capsular

polysaccharide of *Cryptococcus neoformans*) to isolate a large number of peptides from a phage-display library. Four different consensus sequence groups were identified, with the peptides in one predominating over the others in having markedly higher affinities for 2H1.

Our high rate of success in finding cross-reactivity between peptides and carbohydrates may be explained in part by the structures of the Abs involved. In several instances, peptide- and carbohydrate-reactive mAbs have been shown to possess groove-like binding sites (95, 101-106). In contrast, anti-protein Abs directed against discontinuous epitopes on folded proteins have flatter and larger combining sites (107-109), and on average, these Abs isolate weak-binding peptides (30, 110). Work by Vargas-Madrazo *et al.* (111) suggests that the majority of anti-carbohydrate Abs uses a limited subset of canonical CDR structure combinations for the hypervariable loops indicating that this class of Abs uses a restricted structural repertoire, as compared, for example, to the anti-protein Abs.

As a class, anti-carbohydrate Abs have lower affinities for their ligands than do anti-protein Abs. Our anti-carbohydrate mAbs bind their carbohydrate ligands with approximately micromolar affinities, whereas anti-protein mAbs can bind with nanomolar affinities. The lower affinity of anti-carbohydrate mAbs may be related to their polyspecificity; anti-carbohydrate mAbs may bind a range of ligands, from carbohydrates, to peptides, to RNA molecules, with approximately the same affinity. Our data suggest, however, that this polyspecificity would be limited to certain peptides or RNA molecules, only those presenting a surface complementary to the mAb combining

site would be expected to interact with the anti-carbohydrate mAbs. This hypothesis could be investigated by screening RNA libraries with our anti-carbohydrate mAbs to find cross-reactive RNA molecules. Similarly, anti-protein mAbs may interact at low affinities with various ligands. Thus, low affinity anti-protein mAbs may exhibit polyspecificity. In contrast, the lower affinity interactions with different ligands of high affinity anti-protein mAbs may end up being disregarded when compared to the interaction with native antigen.

The relationship between antigenic mimicry and immunologic mimicry by peptides is unclear. Westerink *et al.* (23) demonstrated that a peptide designed from an anti-Id Ab could elicit carbohydrate-reactive Abs, whereas a peptide isolated by Valadon *et al.* (22) failed to elicit carbohydrate-reactive Abs; yet, interestingly, it elicited the correct idiotype, the 2H1 idiotype. Previous work with anti-Id Abs indicates that competition with the target carbohydrate-antigen and high affinity binding are often not sufficient for immunologic mimicry, and at times, may not be necessary. Monafo *et al.* (14) demonstrated that, while only those anti-Id mAbs that competed with carbohydrate antigen for binding to HGAC 39 directly elicited dGAS-reactive Abs, a non-competitive anti-Id mAb induced the greatest enhancement of dGAS reactivity when used along with dGAS for immunization. Importantly, all the anti-Id mAbs against HGAC 39 elicited Abs bearing the HGAC 39-idiotype, (as defined by three anti-Id mAbs) regardless of their ability to compete with antigen. Together, these data (14, 22) suggest that the production of carbohydrate-cross-reactive Abs by anti-Id Abs or peptides may have more to do with eliciting the correct idiotype than with molecular mimicry of the carbohydrate antigen. In

other words, immunogens that elicit Abs capable of binding the original antigen may not carry an "internal image" of the antigen, rather they elicit Abs of the idiotype necessary for recognition of the original antigen. The concept of the internal image implies a conformational similarity between portions of the immunogen and the original antigen. Such conformational similarity may not be necessary in order for the immunogen to generate Abs capable of binding the original antigen. For instance, even though an anti-Id capable of eliciting anti-GAS Abs exists for HGAC 39, HGAC 39 did not isolate a peptide that was a mimic of the carbohydrate epitope since the peptides isolated were not recognized by the other anti-dGAS mAbs. Thus, the immunological mimicry by the anti-HGAC-39 Abs is probably due to recognition of V-domain elements specific to that Ab. Similarly, the putative carbohydrate-mimic peptide designed by Westerink *et al.* (23) could have elicited a carbohydrate-reactive response through Ab-specific interactions rather than carbohydrate mimicry. Importantly, a peptide similar to this one was isolated from a phage-displayed peptide library by the anti-meningococcal-capsular-polysaccharide Ab (personal communication, T. Kieber-Emmons, Univ. of Pennsylvania, Philadelphia).

The data to date suggest that most carbohydrate-cross-reactive peptides may best be used to elicit a specific subset of Abs from within an anti-carbohydrate response. A combined immunization protocol (14, 112), using both the antigen of interest to prime the immune system and the cross-reactive peptide(s) to select or amplify the desired specificities, may be more successful than immunizing with a cross-reactive peptide alone. That is, the immune system could be primed with a carbohydrate based vaccine to

elicit all relevant Abs. Subsequently, the Abs of interest could be amplified with a vaccine containing the appropriate cross-reactive peptide. The boosting peptide vaccine would amplify only those Ab specificities capable of binding both the original carbohydrate antigen and the peptide "enhancer", thus producing an immune response that is more focused to only a few Ab reactivities. Moreover, since carbohydrate antigens are poor immunogens, peptide "enhancers" may boost the immune response against carbohydrate antigens. Alternatively, instead of using cross-reactive peptides to enhance an immune response, they could be used as toleragens to induce anergy in specific B-cell clones, i.e., they could be used to "shut-down" specific Ab producing cells. The apparent increased ability of peptides to discriminate between Ab idiotypes may make them ideal candidates for the induction of anergy in harmful B-cell clones while not affecting the Ab production in protective B-cell clones. By extension, this cross-immunization approach might be extremely useful in enhancing the production of anti-protein Abs against a particular epitope on a target protein antigen, as cross-reactive peptides may not always be structural mimics of discontinuous epitopes (i.e., mimotopes).

The relationship between antigen mimicry and immunologic mimicry by peptides will be clarified by parallel structural analyses of mAbs bound to a target epitope and to cross-reactive peptides. Peptides that are true carbohydrate mimics would interact with the carbohydrate-binding residues in the antigen-binding site. In contrast, peptides that did not elicit carbohydrate-reactive Abs should not interact with the residues critical to carbohydrate binding. Comparative studies of peptide binding to related mAbs would provide support for the idiotype-specific recognition of peptides by anti-carbohydrate

mAbs. A comparison of the residues used to bind either carbohydrates or peptides by a panel of Abs having different idiotypes would help to clarify the nature of the various interactions involved. Unfortunately, the determination of crystal structures for the anti-dGAS mAbs used in this work complexed with their carbohydrate ligands is not practical since the affinities for these ligands is very low. For example, the K_a of Strep 9 binding to a tetrasaccharide or to a hexasaccharide were determined by titration microcalorimetry to be 1.5×10^4 M^{-1} and 3.09×10^4 M^{-1} respectively (29). However, the crystal structures of Se155.4 and SYA/J6 complexed to carbohydrates are already known, and moreover, the carbohydrate binding characteristics of these mAbs have been well studied (88, 95-100). Hence, these are good candidates for studying the mechanisms used by anti-carbohydrate mAbs to bind carbohydrates or peptides. Unfortunately, neither Se155.4 nor SYA/J6 is part of a panel of related mAbs all binding similar epitopes. Thus, it would not be possible to compare directly the binding of peptides by mAbs with different idiotypes.

Clarification of Ab-binding mechanisms will help determine whether, and when, peptides should be used as antigen mimics or as Ab-specific reagents, or both. The rational use of peptides as specific reagents for targeting a limited subset of reactivities within an anti-carbohydrate response would significantly impact their current popularity as potential carbohydrate-mimics for diagnostics, toleragens and vaccines. Currently, peptides are under investigation as structural mimics of carbohydrates (e.g., ref. 23), and as such, could be thought of as antigen-mimics that react with (or immunogen mimics that elicit) all specificities in the response against a carbohydrate immunogen. Our work suggests that carbohydrate-cross-reactive peptides might target a limited subset of

reactivities within an anti-carbohydrate response. This difference should significantly influence strategies for using peptides as substitutes for carbohydrates in diagnostics, toleragens and vaccines.

Peptides or proteins do bind other carbohydrate-reactive proteins. There are only a few studies describing peptides or proteins that bind other classes of carbohydrate-binding proteins, such as lectins (24, 25, 113, 114) and glycosidases (26, 73). At most, three of the many lectins screened so far, E-selectin (114), Concanavalin A (ConA) (24, 25), and IB4 (113), have been shown to bind peptides. However, the peptide isolated by E-selectin did not compete with the oligosaccharide ligand and therefore, probably does not interact with the carbohydrate combining site (26). While the peptides isolated by ConA were able to compete with the carbohydrate ligand (24, 25), recent microcalorimetry titration data indicate that the peptide does not interact with the carbohydrate-binding site (personal communication, E. Toone, Duke University). The behavior of the peptide isolated with IB4, a Gal-α-(1-3)-Gal binding lectin, was different from that of the peptides isolated in our work (113). The peptide isolated by IB4 blocked the binding of IB4 to pig cells expressing Gal-α-(1-3)-Gal on their surface, and its affinity for IB4 was similar to the affinity of α-galactosyl carbohydrates. As well, the peptide blocked the binding of natural human anti-Gal-α-(1-3)-Gal IgG Abs to the pig cells, but its affinity for the Abs was lower than the affinity of carbohydrates. Thus, the peptide was not as specific for its isolating ligate as were the peptides we isolated. The reasons for the difference in peptide reactivity between lectins and anti-carbohydrate mAbs await further investigation; however, a potentially significant difference is that

lectin-binding sites are generally dominated by charged residues, whereas mAb combining sites are composed primarily of hydrophobic and aromatic residues (91).

The binding of peptides to two carbohydrate-reactive enzymes, α-amylase and α-glucosidase, were investigated by Etzkorn *et al.* (73) and Eichler *et al.* (26), respectively. Tendamistat is a protein inhibitor of α-amylase, a hydrolytic enzyme of starch (115). The loop on tendamistat containing the amino acids WRY is believed to be a key element in the interaction between the inhibitor and α-amylase; thus, Etzkorn *et al.* studied peptides containing WRY and different structural constraints. Their data demonstrated that cyclic peptides with a β-turn were the better inhibitors of α-amylase, and only cyclic hexapeptides were able to adopt a β-turn conformation (73).

The work of Etzkorn *et al.* (73) does demonstrate that peptides can bind carbohydrate-reactive enzymes. However, the sequence and conformation of the peptides studied were based upon the known sequence and structure of tendamistat; they were not selected from a library of peptides. Recently, Eichler *et al.* (26) used synthetic combinatorial libraries (SCLs) to identify a peptide inhibitor of α-glucosidase. SCLs are made synthetically on resin; consequently, SCLs can contain unnatural amino acids (116). This work with α-glucosidase used a panel of libraries. The libraries varied the number of random positions from five to seven, contained all L-amino acids, all D-amino acids or combinations of both, were cyclic due to the presence of disulfides or lactams, or were the linear analogs. Out of all the libraries, the most active was a cyclic lactam library containing both D- and L-amino acids; the library had the structure cyclo(xXxXxN), where X designates one of the nineteen L-amino acids (cysteine is

excluded) and x designates one of the nineteen D-isomers. In order to determine the sequences of the active components, an iterative synthesis and screening process was performed. For the first iteration, nineteen sub-libraries were synthesized with the first position containing a fixed amino acid. For each iteration, one more position was fixed until the entire amino acid sequence had been determined. Thus, the amino acid sequence was determined one position at a time, and cyclo(iWyRyN) was identified as the most active peptide in the original library. The K_i of this peptide for α-glucosidase was determined to be 1.7 μM, compared to 6.75 μM for *N*-methyldeoxynorjirimycin. Moreover, the peptide was shown to be selective for α-glucosidase over β-glucosidase (26).

3.4 Materials and Methods

The peptide, NH_2ADGADRPVPYGACGOrn (biotin)-NH_2 (DRPVPY-peptide), was synthesized and HPLC-purified by the Alberta Peptide Institute (Edmonton, Alta.). The anti-GAS CWPS mAbs, SA-3 (3), Strep 9, (40) (a generous gift from J. B. Pitner, Becton Dickinson Research Center, Durham, NC), HGAC 39.G3 (4), HGAC 47.G3 (4), HGAC 85.G3 (4) and HGAC 101.G3 (4) (kindly provided by N. S. Greenspan, Case-Western University School of Medicine, Cleveland, OH), were raised against a heat-killed, pepsin-treated GAS (dGAS) vaccine (85). SA-3 is an IgM, and the others are IgG_3; all use kappa light chains. The dGAS used in this work was kindly provided by J. B. Pitner and D. R. Bundle (University of Alberta, Edmonton). The production and characterization of the pcAbs were described previously (3). mAbs Se155.4 and SYA/J6

(kindly provided by D. R. Bundle) were raised against *Salmonella* serogroup B and *Shigella flexneri* Y, respectively. The amino acid sequences are published for mAbs Se155.4 (88), HGAC 39, HGAC 47 and HGAC 101 (4), and those of Strep 9, SA-3, and SYA/J6 are published in reference 29. The syntheses of the GAS oligosaccharides and glycoconjugates have been published (3, 36, 89, 90). The lipopolysaccharides and oligosaccharides of *Salmonella* serogroup B and *Shigella flexneri* Y were generous gifts from D. R. Bundle.

Eleven different peptide libraries, displayed as fusions to cpVIII of the phage vector f88.4, and their screening with mAbs (HGAC 39, HGAC 47, HGAC 101, Strep 9, Se155.4 and SYA/J6) have been described, as well as the isolation and analysis of phage clones (30). Hexamer (60) and 15-mer peptide libraries (117), displayed as fusions to cpIII of the phage vector fUSE5, were screened by SA-3 and the pcAbs as described (30). The 15-mer library (117) was kindly provided by H. Saya (Univ. of Kumamoto, School of Medicine, Japan). Brief descriptions of biopanning, titering and DNA sequencing are given below.

3.4.1 Biopanning.

Aliquots containing 10^{11} to 10^{12} virions from each phage-displayed library were affinity selected (biopanned) on biotinylated mAbs that had been immobilized in microwells coated with avidin or streptavidin (1 μg) (30). For the first round of biopanning 1 μg of mAb was used, while 0.1 μg was used in each subsequent round. For each round of biopanning, the microwells containing immobilized mAb were incubated

with aliquots of the phage for 4 h at 4°C. After washing away unbound phage, the bound phage were eluted with a low pH buffer (0.1 N HCl adjusted to pH 2.2 with glycine, and 1 mg/mL BSA). After neutralizing the eluate, the phage were amplified. *E. coli* K91 cells were used to amplify the phage; the K 91 cells were infected with the eluted phage for 15 min at RT. After inducing tetracycline resistance by the addition of super broth containing 0.2 μg tetracycline/mL, the cells were grown overnight at 37°C. The amplified phage were used in the next round of biopanning. Enrichment for Ab-binding phage was assessed by titering after each round of panning (see below). Enriched, amplified phage pools were tested for binding by ELISA after the third and fourth rounds of screening. Ten individual clones were isolated from the two or three pools of enriched phage displaying the highest enrichment and/or ELISA signal. The clones were analyzed by ELISA (described in reference 60 and below) and their displayed peptide sequences were determined (described in reference 118 and briefly below).

3.4.2 Titering.

A spot titering technique was used to determine the enrichment for binding phage after reach round of biopanning. Two microliter aliquots of infected (non-amplified) K91 cells were serially diluted 10-fold and 20 μL were spotted onto NZY plates containing 20 μg tetracycline/mL. Control samples contained known amounts of f88.4 virions. After an overnight incubation at 37 °C, the number of phage present in a sample were determined. The phage enrichment was calculated as the number of virions eluted divided by the number of virions added to the biopanning experiment.

3.4.3 DNA sequencing.

The displayed peptide sequence was determined by dideoxynucleotide sequencing, (119) loosely following the procedure of Haas and Smith. (118). Aliquots of phage (3 μL) were incubated for 10 min at room temperature with NaOH (3 μL, 180 mM) containing 10 ng of f88.4 or fd-tet sequencing primer. (The sequencing primer was end-labeled by mixing 45 pmol of the 5'-OH oligonucleotide primer with 200 μCi of [γ-^{32}P]ATP, 1 μL of 10x kinase buffer [0.5 M Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM dithioerythritol, 1 mM spermidine, 1 mM Na_2EDTA, pH 8], 8 units of T4 polynucleotide kinase in a total volume of 10 μL, and incubating at 37 °C for 15 min. The enzyme was inactivated by adding EDTA (140 μL) and heating for 70 °C for 15 min.) After the phage were incubated with the primer, the mixture was neutralized with a neutralizing buffer (3 μL, 0.173 N HCl, 192 mM Tris-HCl, pH 7.2, 34 mM D,L-isocitrate, pH 7.2, 11.3 mM $MnCl_2$, 85 μM phenol red) and incubated at 37 °C for 5 min. Two, 3 μL samples of each primed template were placed into microwells.

T7 DNA polymerase was added to the R and Q termination mixes to give a concentration of 0.086 units/μL. (The R termination mix contains 67 μM dNTPs, pH 7, 16.7 mM Tris-HCl, pH 7.5, 66.7 mM NaCl, 13.3 mM dithioerythritol, 100 μg/mL BSA, 3.2 μM ddATP, 3.2 μM ddGTP, 0.32 μM ddCTP. The Q termination mix contains 67 μM dNTPs, pH 7, 16.7 mM Tris-HCl, pH 7.5, 66.7 mM NaCl, 13.3 mM dithioerythritol, 100 μg/mL BSA, 3.2 μM ddATP, 3.2 μM ddTTP, 0.32 μM ddCTP.) The R termination mix (3 μL) was added to half the samples while the Q termination mix (3μL) was added

to the other half of the samples. The samples were incubated at 37 °C for 5-10 min and the polymerization reactions were terminated by adding formamide load buffer (4 μL) to each well. The samples were analyzed by gel electrophoresis.

3.4.4 ELISAs.

All washes were performed with TBS (50 mM TrisHCL, 150 nM NaCl, pH 7.5) and 0.1% Tween 20. Except where noted, wells were blocked with blotto (200 μL, 5% milk powder in TBS) for 2 h at 4°C, IgG Abs were used at 100 nM in 35 μL of blotto, and the IgM (SA-3) was used at 20 nM in 35 μL of blotto; incubation times were 4 h at 4°C. Biotinylated mAbs were detected with avidin:horseradish-peroxidase complexes (30), and non-biotinylated mAbs and pcAbs were detected with secondary Abs conjugated to horseradish peroxidase (Pierce, Rockford, IL). Optical densities are reported as $(A_{405}-A_{490})$ x 1000 (30).

Phage ELISAs were performed as described previously (30), using sample phage or controls without peptide inserts (fd-tet (60) or f88.4 (30)). Briefly, microwells were coated overnight at 4°C with anti-phage Ab (1 μg in 35 μL of TBS). After blocking and washing three times, 10^{10} virions in 35 μL of TBS were added to the wells and incubated for 2 h at 4°C (30). Biotinylated Abs were added after three washes and incubated for 4 h at 4°C.

For the peptide ELISA, microwells were coated overnight with streptavidin (1 μg in 35 μL of TBS), and blocked. The wells were then washed and incubated with the DRPVPY-peptide (4nM) or TBS (40 μL) for 30 min at room temperature. The remaining

streptavidin sites were blocked by incubating with biotin (1.5 mM) for 1 h at 37°C. The biotinylated mAb was added after three washes and incubated for 4 h at 4°C.

For competition ELISAs, the immobilized antigens were either phage, a synthetic glycoconjugate, or the native carbohydrate antigen (dGAS or a lipopolysaccharide) that had been immobilized by adsorption, or the DRPVPY-peptide immobilized in streptavidin-coated microwells. Competition was established by the addition of equal volumes of an Ab and an inhibitor to antigen-coated or control microwells. When synthetic oligosaccharides were used as inhibitors, microwells were coated overnight at 4°C with 35 μL of 0.1 M bicarbonate buffer, pH 9.0, containing $4x10^6$ dGAS (for mAbs HGAC 39 and Strep 9) or $4x10^7$ dGAS (for mAbs SA-3, HGAC 47 and HGAC 101), then blocked with BSA. After washing the blocked wells three times, increasing concentrations of the oligosaccharide inhibitor were added to wells along with a constant concentration of mAb. mAb SA-3 and biotinylated HGAC mAbs were used at 10 nM; Strep 9 (not biotinylated) was used at 20 nM. Bound mAb was detected after overnight incubation at room temperature, followed by six washes.

3.4.5 Microcalorimetry.

A solution of SA-3 was titrated with either the DRPVPY-peptide or a hexasaccharide, Hexa 2 (9), using a Microcal Omega titration microcalorimeter (120) and methods described previously (121). In each case a solution of the mAb was titrated with ligand in 2-5 mL aliquots. The concentration of SA-3 was such that the product of the concentration of binding sites and the binding constant was in the range of 1 to 1000.

The ligand concentration in the syringe was such that the final ligand concentration was at least 10 x K_d. On each injection an exothermicity was recorded. For the SA-3:Hexa 2 interaction, a cell with a volume of 1.3678 mL was loaded with 68.4 μM SA-3 dissolved in 10 mM Na_2PO_4, 127 mM NaCl, pH 7.0, and 1.25 mM Hexa 2, dissolved in the same buffer, was added in sixteen, 15 μL aliquots at 37°C. For the SA-3:DRPVPY-peptide interaction, 20.5 μM SA-3 was injected with 10 μL aliquots of 0.168 mM DRPVPY-peptide. All injections were 5 s with 3 min intervals between injections; the data were analyzed as described (121).

CHAPTER 4

INVESTIGATIONS OF THE MOLECULAR REQUIREMENTS FOR ANTIBODY-MEDIATED PEPTIDE-CARBOHYDRATE CROSS-REACTIVITY

4.1 Introduction

The work described in this Chapter was performed to investigate further the molecular basis of Ab-mediated cross-reactivity between carbohydrate and protein antigens. The goal was to determine which amino acids were important for the cross-reactivity of the peptides isolated in Chapter 3 and the chemical requirements at these positions for binding carbohydrate-reactive mAbs. These investigations were used to select a peptide for use in more detailed studies of its interaction with a carbohydrate-reactive mAb. Comparative studies of mAb:peptide complexes and mAb:carbohydrate complexes in the future would help to clarify the mechanisms of cross-reactivity.

Chiron's Multipin™ (San Diego, CA) peptide synthesis kit was used for the investigations of the molecular requirements of peptide cross-reactivity (31-35). The kit provides an easy and economical means of synthesizing and testing many peptides at once. For approximately the same cost as having one peptide commercially synthesized, ninety-six different sequences can be synthesized in quantities large enough for preliminary, survey-type studies. Thus, small peptide libraries based upon the parent peptide sequences isolated in Chapter 3 were tested to determine the residues critical for binding, as well as the chemical requirements for binding at these key positions.

A summary of the key findings is provided prior to a presentation of the detailed results. The first question to be answered was whether the peptides would be recognized when not in the context of the large phage coat-protein. Consequently, the cross-reactive parent peptide sequences isolated by all the anti-dGAS mAbs studied in Chapter 3, Se155.4 and SYA/J6 were first synthesized with the Multipin™ kit. While the mAbs bind their corresponding peptides with a signal greater than a blank pin, little specificity for their corresponding peptide was observed. Thus, the mAbs appear to be able to bind peptides on pins, but the interaction is different from when the peptides were displayed on phage. The peptides synthesized on pins must behave as the peptides displayed on phage in order for the results to apply to the reactivities observed in Chapter 3. The reasons for the difference in peptide activity depending upon their presentation are unclear. Next, the residues critical for the binding of peptides synthesized on pins were determined by studying alanine-substitution analogs of several of the parent peptides; each residue was substituted with alanine, one at a time (33, 122). A residue was determined to be important for recognition if mAb binding was significantly decreased, or abolished, upon alanine replacement of the residue. The results showed that the critical residues for recognition of the SA-3-isolated sequence, AD**GA**D**RP**VPYGAC**G**, were the first glycine, the second alanine, the arginine, the first proline and the final glycine (shown in bold). These critical residues are somewhat comparable to the conserved residues observed for the sequences isolated from the biopanning experiments in Chapter 3. The consensus sequence determined for SA-3-isolated peptides was **RPXXY** (Chapter 3); thus, the arginine and proline, from the variable region of the

phage-displayed peptides, were shown to be important for the interaction between peptides on pins and SA-3. The key amino acids for recognition of the SYA/J6-isolated sequence, **M**D**WN**MH**AA**EGDD, were the first methionine, the tryptophan, the asparagine and both alanines (shown in bold). In this instance, the critical residues for SYA/J6 binding of a peptide on a pin were different from the conserved residues observed for the sequences isolated from the biopanning experiments in Chapter 3; the consensus sequence determined for SYA/J6 isolated peptides was **NM** (Chapter 3). Peptides corresponding to smaller portions of the parent sequences were also tested in order to determine the minimum length required for mAb recognition. It appeared that an octamer was the shortest peptide recognized by either SA-3 or SYA/J6. Finally, the chemical requirements for binding at the key positions were investigated by studying the activity of many replacement analogs of the parent sequences. Those residues shown to be important by alanine-replacement were replaced by several other amino acids. The chemical properties of the amino acids used for the replacements were diverse; thus, insight into the chemical properties required at key positions for mAb recognition was gained. For instance, the requirement for arginine and proline in the SA-3-isolated sequence, ADGAD**RP**VPYGACG, was shown to be absolute; the requirement for tryptophan, asparagine and the first alanine in the SYA/J6-isolated sequence **M**D**WN**MH**AA**EGDD was absolute, whereas the requirement for methionine or the second occurrence of alanine was less strict.

Based on all the analog work, one sequence was chosen for larger scale synthesis in order to study further its binding to an anti-carbohydrate mAb. The sequence

MDWNMHAA was synthesized as a free molecule, since it was bound by SYA/J6 when synthesized on a pin. A SYA/J6-reactive peptide was chosen since there is much information available concerning the interaction of SYA/J6 with carbohydrates to which the interaction of SYA/J6 with a peptide could be compared (98, 100). The sequence MDWNMHAA was chosen specifically since it was the shortest peptide tested which SYA/J6 bound and it was not too hydrophobic. SYA/J6 was shown to recognize the peptide in solution and microcalorimetry studies of the mAb:peptide interaction were performed. Moreover, co-crystallization of the Fab fragment of SYA/J6 with the peptide and TRNOE NMR studies with the Fab fragment and the peptide are being attempted. The structure of the mAb:peptide complex could be compared to that of a mAb:carbohydrate complex to gain additional insight into the nature of mimicry.

4.1 Results

4.1.1 Synthesis of peptides on pins to determine whether binding to anti-carbohydrate mAbs would still occur once the peptides were no longer displayed on phage.

The sequences chosen for synthesis were those investigated in Chapter 3 for their specificity (Table 3.5). As shown in Table 4.1, two peptides isolated by each of the anti-dGAS mAbs, Strep 9, HGAC 39, HGAC 47, and HGAC 101, were synthesized. The HGAC 39-isolated sequence ATYRPVPAEFARKHL was not studied in Chapter 3, but was synthesized in order to have two HGAC 39-isolated sequences. The only SA-3-isolated sequence synthesized, ADGADRPVPYGACG, corresponded to that of the

DRPVPY-peptide studied in Chapter 3. Three peptides isolated by either Se155.4 or SYA/J6 were synthesized. Again, the Se155.4-isolated sequence MYPMSHAAEGDD was not studied in Chapter 3, but was synthesized in order to have three Se155.4-isolated sequences.

Peptides made with the Multipin™ peptide synthesis kit can be as long as 15 to 20 amino acids (123). Thus, it was possible to add extra amino acids, which corresponded to residues of the adjacent cpVIII, to the C-terminal ends of the shorter peptide sequences isolated by mAbs, HGAC 101, Se155.4 and SYA/J6. These additional amino acids were included since the effect of the flanking regions surrounding a peptide insert upon Ab recognition was unknown. However, additional amino acids could not be added to the longer sequences isolated by mAbs SA-3, Strep 9, HGAC 39, HGAC 47, and one by Se155.4 since the fidelity of the final products would be compromised.

In general, the cross-reactive peptides were bound, above the background level of a blank pin, by the mAbs used for their isolation, but the specificity of the mAbs for their corresponding peptides was low. As shown in Table 4.1, all of the HGAC mAbs bound their corresponding peptides above the background level of a blank pin. However, Strep 9 did not bind either of the Strep 9-isolated peptides above background levels. The lack of Strep 9 binding to the peptides on pins is interesting when compared to Strep 9's strong binding to the phage-displayed peptides (Table 3.5). It may be that cpVIII affects the conformation of these particular peptides; amino acids corresponding to the residues of the adjacent cpVIII were not included for these peptides since they were isolated from libraries containing long random peptide inserts. Furthermore, the formation of disulfide

bonds was not controlled in these syntheses. A lack of the required disulfide bonds could affect the peptides' activities (Table 3.11). Similarly, the strong binding of HGAC 47 to the peptides on pins is intriguing when compared to HGAC 47's weak binding to the phage-displayed peptides (Table 3.5). In this instance, it may be that the higher density of peptides on the pins increases the avidity of the HGAC 47:peptide interaction as compared to the lower density of peptides displayed on the phage (32, 33, 60, 66).

The activity of the peptides with the anti-dGAS mAbs not used for their isolation is interesting. The specificity of a mAb for its isolated sequence, as well as the pattern of activity with the other cross-reactive peptides on pins is different from when the peptides were displayed on phage (Table 3.5). The data in Table 4.1 show that HGAC 39 bound the SA-3-isolated sequence when it was displayed on a pin, whereas none of the other anti-dGAS mAbs were able to bind this peptide when it was free in solution (Table 3.5) Furthermore, the data in Table 4.1 show that all the anti-dGAS mAbs bind the Strep 9-isolated peptide MCPPLYSPSACA, even though Strep 9 itself does not bind this peptide. When this peptide was displayed on phage, SA-3 was the only other mAb that bound (Table 3.5). While the pin presentation may have destroyed Strep 9 binding, the increased density of the pin presentation may have increased the avidity of this peptide's interaction with the other anti-dGAS mAbs (32, 33, 60, 66). Increased avidity may explain why SA-3, an IgM, was the only mAb to bind this peptide when it was displayed on the phage and why SA-3 had the strongest binding to this peptide when it was synthesized on a pin. The other Strep 9-isolated peptide, YPYCGHALCPGLYADAS, was not bound by any of the other anti-dGAS mAbs whether displayed on pins or on

phage (Table 3.5). While SA-3 did bind the HGAC 39-isolated peptide, ADAAPSPTPYLPRLS, when it was displayed on phage (Table 3.5), SA-3 did not bind this sequence when it was displayed on a pin (Table 4.1). However, SA-3 did strongly bind the other HGAC 39-isolated sequence, ATYRPVPAEFARKHL (Table 4.1); the specificity of this peptide, when displayed on phage, was not tested. The HGAC 47-isolated sequences were specific for HGAC 47 whether displayed on pins or on phage (Table 3.5), but HGAC 47 had high background binding to most of the other peptides. Finally, the data in Table 4.1 show that neither HGAC 39 nor HGAC 47 bound the two HGAC 101-isolated peptides, even though they did when these peptides were displayed on phage (Table 3.5). Thus, the pattern of activity for the anti-dGAS mAbs for the peptides on pins was different from the pattern of activity observed for the phage-displayed peptides.

Table 4.1 also shows the activity of the peptides corresponding to the sequences isolated by Se155.4 and SYA/J6. As expected, Se155.4 did not bind any of the peptides synthesized on pins since Se155.4's interaction with the phage-displayed peptides was weak (Table 3.2). In contrast, SYA/J6 bound two of its corresponding peptides. As was expected based upon the work with phage-displayed peptides (Table 3.5), none of the anti-dGAS mAbs bound the sequences isolated by Se155.4 or SYA/J6, and neither did Se155.4 nor SYA/J6 bind any of the anti-dGAS-isolated peptides.

Table 4.1. Anti-carbohydrate mAb binding to synthetic peptides whose sequences correspond to those isolated in Chapter 3.

	mAb							
Peptide sequence	SA-3	Strep 9	HGAC 39	HGAC 47	HGAC 101	Se155.4	SYA/J6	+/-[a]
ADGADRPVPYGACG[b]	**1135**[c,d]	97	613[e]	245	n/d[f]	n/d	114	-
MCPPLYSPSACA	540	**93**	278	307	237	104	73	-
YPYCGHALCPGLYADAS	111	**124**	150	190	166	101	78	-
ADAAPSPTPYLPRLS	142	96	**146**	183	148	**84**	74	-
ATYRPVPAEFARKHL	1381	91	**220**	95	83	**87**	69	n/a[g]
MCRPGIPTHHCA	160	97	130	**814**	153	103	69	+
DCGNMRQAEVCR	131	101	172	**232**	169	92	73	+
SCISAACFCIGGPAE	97	88	125	184	**143**	86	82	-
KQLMAPAAEGDD	118	97	136	158	**139**	90	109	-
NYPMDHAAEGDD	117	92	125	127	135	**86**	107	-
EPYPMSEANYVRPMP	124	101	166	199	156	**100**	86	-
MYPMSHAAEGDD	126	96	167	180	162	**95**	97	n/a
MDWNMHAAEGDD	106	129	117	202	130	71	**140**	+
MGVICMNMECDRNMPAE	110	117	166	188	139	80	**68**	-
YTTQCGYGGCMNFEPAE	134	99	146	177	127	104	**176**	+
blank pin	142	100	84	108	98	112	79	
+/-[a]	-	-	-	-	-	-	+	

a) a comparison of the pattern of activity, in the row or column, to the corresponding row or column in Table 3.5. + indicates the pattern of activity is the same, - indicates that the pattern of activity is different.

b) underlined sequences indicate unique consensus groups

c) $(A_{405}-A_{490}) \times 1000$

d) numbers in bold indicate the clone was detected by the corresponding mAb (e. g., SA-3 isolated the sequence ADGADRPVPYGACG).

e) underlined numbers indicate significant cross-reactivity with a mAb not used to selected the sequence

f) not determined

g) not applicable

4.1.2 Choice of four peptides for further investigation using the Multipin™ kit.

Investigations into the residues critical for the binding of cross-reactive peptides to anti-carbohydrate mAbs were performed for a few sequences. The DRPVPY-peptide isolated by SA-3 (ADGADRPVPYGACG) was investigated since it was bound well by, and was specific for SA-3 (Chapter 3, ref. 29). The Strep 9-isolated peptide, MCPPLYSPSACA, was chosen due to its interesting mAb binding pattern (Table 4.1); even though Strep 9 did not bind this peptide when synthesized on a pin, all the other anti-dGAS mAbs bound this peptide. The HGAC 39-isolated peptide, ATYRPVPAEFARKHL, was chosen since it was bound by both HGAC 39 and SA-3 (Table 4.1). Finally, a SYA/J6-isolated peptide was chosen for investigation since a crystal structure of this mAb complexed with a carbohydrate is known (98, 100); the sequence MDWNMHAAEGDD was chosen rather than YTTQCGYGGCMNFEPAE Y since the former sequence is shorter and does not have the potential for disulfide bonding. While the formation of disulfide bonds can be helpful since they reduce conformational flexibility, they complicate the synthesis.

4.1.3 Synthesis of analogs in which each amino acid was replaced, one at a time, with either alanine or tyrosine (33, 122).

Peptides were synthesized, with help from Paula Brown, in order to determine the residues important for mAb recognition; an amino acid was considered to be important if its substitution by a different amino acid significantly affected the activity of the peptide

(31, 33, 35). Alanine was the main choice for replacement since it does not have a side chain beyond the β carbon, yet it does not alter the main-chain conformation as proline or glycine might. Moreover, alanine does not impose extreme electrostatic or steric effects, and it is frequently found both buried within and exposed on the surface of proteins (124-126). However, using alanine to replace alanine, serine or glycine would provide little to no information since alanine, serine and glycine are frequently self-replacing (33), that is the small side chains of these three amino acids allows them to be substituted by one another with little effect upon recognition phenomena. To overcome this problem, alanine, serine and glycine were replaced with tyrosine since it has a very different side chain and can also be found in proteins either buried or exposed (124).

The sets of peptides based upon the HGAC 39-isolated and Strep 9-isolated parent peptides were not successful. As can be seen from the data in Table 4.2, none of these peptides, including the control parent peptides, were recognized by their respective mAbs. The lack of HGAC 39 activity is in contrast to the data in Table 4.1 and may be the result of incomplete coupling of arginine or of the amino acids following arginine. The chemically-protected side chain of arginine is very large and may sterically hinder its coupling or the coupling of the next amino acid. It may be that the coupling reaction was not given enough time to go to completion in this synthesis. Nonetheless, the HGAC 39-isolated sequence was not investigated further. The set of peptides based upon the Strep 9-isolated sequence was synthesized with the thought that some activity, or variation in activity, may be seen for the different sequence analogs. However, only one analog

exhibited any activity (Table 4.2); thus, the Strep 9-isolated sequence was not investigated further.

Table 4.2. Binding of HGAC 39 and Strep 9 to the alanine-substitution analogs of their respective parent peptide sequences.

Peptide sequence	HGAC 39	Peptide sequence	Strep 9
ATYRPVPAEFARKHL	46[a]	MCPPLYSPSACA	48
YTYRPVPAEFARKHL	49	ACPPLYSPSACA	47
AAYRPVPAEFARKHL	51	MAPPLYSPSACA	57
ATARPVPAEFARKHL	42	MCAPLYSPSACA	51
ATYAPVPAEFARKHL	51	MCPALYSPSACA	51
ATYRAVPAEFARKHL	51	MCPPAYSPSACA	44
ATYRPAPAEFARKHL	49	MCPPLASPSACA	45
ATYRPVAAEFARKHL	54	MCPPLYYPSACA	44
ATYRPVPYEFARKHL	50	MCPPLYSASACA	48
ATYRPVPAAFARKHL	45	MCPPLYSPYACA	45
ATYRPVPAEAARKHL	40	MCPPLYSPSYCA	55
ATYRPVPAEFYRKHL	47	MCPPLYSPSAAA	353
ATYRPVPAEFAAKHL	45	MCPPLYSPSACY	48
ATYRPVPAEFARAHL	56		
ATYRPVPAEFARKAL	64		
ATYRPVPAEFARKHA	47		
blank pin	51	blank pin	51

a) $(A_{405}-A_{490}) \times 1000$

The data in Table 4.3 suggested that the residues important for binding in the SA-3-isolated sequence were the first glycine, the second alanine, the arginine, the first proline and the final glycine (AD**GA**D**RP**VPYGAC**G**). The separate substitution of both the first glycine and the second alanine by tyrosine significantly reduced SA-3 binding, but not to background levels. The separate substitution of both the arginine and the first proline by alanine and the substitution of the final glycine by tyrosine eliminated SA-3 binding. Alanine (or tyrosine) substitution of the other amino acids did not affect SA-3

binding. Therefore, the data in Table 4.3 suggest that the first glycine, and the second alanine were not as critical for binding as the arginine, the first proline and the final glycine.

Table 4.3. The binding of SA-3 and the other anti-dGAS mAbs to the alanine-substitution analogs of the peptide corresponding to the sequence isolated by SA-3

	mAb				
Peptide sequence	SA-3	Strep 9	HGAC 39	HGAC 47	SYA/J6
ADGADRPVPYGACG	1135[a]	97	613[b]	245	114
YDGADRPVPYGACG	931	82	295	255	146
AAGADRPVPYGACG	1674	219[c]	238	230	109
ADYADRPVPYGACG	**242**[d]	85	222	317	236
ADGYDRPVPYGACG	**432**	82	206	277	233
ADGAARPVPYGACG	1348	104	416	240	116
ADGADAPVPYGACG	**60**	73	185	361	113
ADGADRAVPYGACG	**49**	67	142	274	145
ADGADRPAPYGACG	945	83	142	234	121
ADGADRPVAYGACG	705	73	140	258	154
ADGADRPVPAGACG	1921	91	166	248	250
ADGADRPVPYYACG	1515	84	244	184	170
ADGADRPVPYGYCG	1331	91	946	189	103
ADGADRPVPYGAAG	1588	140	1587	203	125
ADGADRPVPYGACY	**72**	71	450	179	98
blank pin	51	71	84	202	95

a) $(A_{405}-A_{490}) \times 1000$
b) underlined numbers indicate significant cross-reactivity with a mAb not used to select the sequence
c) may be residual binding
d) numbers in bold indicate a significant decrease in SA-3 binding as compared to SA-3 binding to the parent peptide sequence.

These results confirm the importance of arginine and proline to the consensus sequence, **RPXXY**, deduced from the sequences isolated by biopanning (Table 3.2); however, tyrosine was not indicated to be important. Analysis of the sequences isolated by biopanning could not suggest a role for the first glycine and the second alanine since

these residues were within the constant cpIII region of the phage (60). It may be that these two residues aid the formation of the correct peptide conformation. The apparent importance of the final glycine is very surprising since it was not part of the phage-displayed peptide isolated (29, 33). This amino acid was included on the synthetic DRPVPY-peptide merely as a structureless extension of the C-terminus and was, therefore, not selected by SA-3 during the biopanning.

The ability of the other anti-dGAS mAbs to bind the alanine-substitution analogs of the DRPVPY-peptide was tested. As can be seen in Table 4.3, the anti-dGAS mAbs Strep 9 and HGAC 47 did not bind the DRPVPY-peptide on a pin, similar to their behavior with the DRPVPY-peptide free in solution (Table 3.5). (The binding of HGAC 101 to this peptide sequence synthesized on a pin was not tested because there were only limited amounts of this mAb available in our laboratory.) Moreover, Strep 9 and HGAC 47 did not bind the alanine-substitution analogs. However, HGAC 39 did bind the parent peptide sequence, in contrast to its behavior with the synthetic DRPVPY-peptide (Table 3.5), and it also bound some of the alanine-substitution analogs (Table 4.3). In general, the level of activity was low and HGAC 39's binding was not as affected by alanine substitution, as was the binding of SA-3, suggesting that HGAC 39's binding was nonspecific.

The data in Table 4.4 suggested that the important residues for binding in the SYA/J6-isolated sequences were the first methionine, the tryptophan, the asparagine and both alanines (**M**D**WN**MH**AA**EGDD). The separate substitution of the first methionine by alanine and the second alanine by tyrosine significantly reduced SYA/J6 binding, but

not to background levels. The separate substitution of the tryptophan, and the asparagine by alanine, as well as the first alanine by tyrosine, eliminated SYA/J6 binding. Alanine (or tyrosine) substitution of the other amino acids did not affect SYA/J6 binding. Therefore, the data in Table 4.4 suggest that the first methionine and the second alanine were not as critical for binding as the tryptophan, the asparagine and the first alanine. These results differ from the consensus sequence suggested by the sequences isolated by biopanning; the consensus sequence was suggested to be only **NM**.

Table 4.4. SYA/J6 binding of alanine-substitution analogs of the peptide corresponding to the SYA/J6-isolated sequence.

	mAb			
Peptide sequence	SYA/J6	Strep 9	HGAC 39	HGAC 47
MDWNMHAAEGDD	1337[a]	74	93	166
ADWNMHAAEGDD	**596**[b]	75	76	204
MAWNMHAAEGDD	1344	93	85	414
MDANMHAAEGDD	**52**	76	75	184
MDWAMHAAEGDD	**52**	66	70	148
MDWNAHAAEGDD	899	67	80	137
MDWNMAAAEGDD	762	77	81	182
MDWNMHYAEGDD	**42**	71	62	159
MDWNMHAYEGDD	**447**	60	66	159
MDWNMHAAAGDD	1112	68	86	157
MDWNMHAAEYDD	996	66	72	132
MDWNMHAAEGAD	930	69	73	155
MDWNMHAAEGDA	983	78	84	167
blank pin	95	71	84	202

a) $(A_{405}-A_{490}) \times 1000$

b) numbers in bold indicate a significant decrease in SYA/J6 binding as compared to SYA/J6 binding to the parent peptide sequence.

The binding of the anti-dGAS mAbs to the alanine-substitution analogs of the SYA/J6-isolated peptide was tested. As expected, none of these mAbs bound any of

these peptides (Table 4.4). Furthermore, SYA/J6 did not bind the alanine-substitution analogs of the DRPVPY-peptide (Table 4.3).

4.1.4 Synthesis of peptides corresponding to smaller portions of the parent peptides on pins in order to determine the minimum length required for recognition (33).

Table 4.5 shows the sequences used to determine the minimum peptide size recognized by SA-3; each sequence was shortened by two amino acids from the C-terminus and half of the sequences did not include the *N*-terminal amino acids alanine and aspartic acid.

Table 4.5. SA-3 binding to shortened analogs of the peptide corresponding to the SA-3-isolated sequence.

Peptide sequence	$(A_{405}-A_{490}) \times 1000$
ADGADRPVPYGACG	1615
ADGADRPVPYGA	1298
ADGADRPVPY	1412
ADGADRPV	1736
ADGADR	**52**[a]
GADRPVPYGACG	1156
GADRPVPYGA	759
GADRPVPY	456
GADRPV	**49**
background	55

a) numbers in bold indicate that SA-3 binding was not above background levels.

The data in Table 4.5 illustrate that, for the peptides possessing the *N*-terminal alanine and aspartic acid, a total of six amino acids could be deleted from the *C*-terminus without changing SA-3 binding. However, deletion of eight amino acids from the *C*-terminus abolished SA-3 binding. The deletion of eight amino acids included the

deletion of the proline; thus, it was not surprising that activity was abolished since the proline was shown to be critical for SA-3 recognition (Table 4.3). The data also illustrate that when the *N*-terminal alanine and aspartic acid were deleted, SA-3 binding decreased and a total of only four *C*-terminal amino acids could be deleted. SA-3 binding to the peptides with either two or four *C*-terminal residues deleted was above background. However, deletion of six *C*-terminal residues eliminated SA-3 binding, even though all of these residues were replaceable with alanine (Table 4.3), and they could be deleted if alanine and aspartic acid were included at the *N*-terminus. With or without all the *N*-terminal amino acids, the shortest peptides bound by SA-3 were eight amino acids long. This suggests that eight amino acids are required to form a conformation that correctly presents critical binding residues. However, a heptapeptide may be able to present correctly critical binding residues. The sequence ADGAD**RP** which contains the arginine and proline shown to be critical for SA-3 recognition, unlike the hexapeptide ADGAD**R**, was not tested.

The peptides synthesized to determine the minimum length required for SYA/J6 recognition are shown in Table 4.6. Each peptide was shortened by two amino acids from the *C*-terminus; all of the *N*-terminal amino acids were always included since the first residue was shown to be important for SYA/J6 recognition (Table 4.4). The data in Table 4.6 illustrate that a total of four *C*-terminal residues could be deleted; in fact, SYA/J6 binding increased with the deletion of these amino acids. However, deletion of six *C*-terminal amino acids, which included the two alanines shown to be important for SYA/J6 recognition, eliminated SYA/J6 binding.

Table 4.6. SYA/J6 binding to shortened analogs of the peptide corresponding to the SYA/J6-islated sequence.

Peptide sequence	$(A_{405}-A_{490}) \times 1000$
MDWNMHAAEGDD	754
MDWNMHAAEG	1009
MDWNMHAA	908
MDWNMH	**37**[a]
MDWN	**88**
background	55

a) numbers in bold indicate that SYA/J6 binding was not above background levels.

4.1.5 Synthesis of substitution analogs of the parent peptides isolated by SA-3 or SYA/J6 in order to determine the chemical requirements at the key positions (31, 33, 35).

The SA-3- and SYA/J6-isolated peptides were chosen for further study since these peptides were well-behaved (see Table 4.1). We would have liked to investigate the HGAC 39-isolated peptide, but this peptide was not well behaved (see Tables 4.1 and 4.2). In addition, the SA-3-isolated peptide was well studied (Chapter 3) and the SYA/J6-isolated peptide would be important for future investigations; the SYA/J6-peptide complex could be compared to the SYA/J6-carbohydrate complexes already studied.

The pin technology can be used to determine the chemical requirements at a position by synthesizing peptides having every amino acid substituted, one at a time, by every other amino acid (31, 33, 35). Since the side chains of the amino acids span a wide variety of chemical functionalities (e.g., hydrophobic, hydrophilic, charged, uncharged, polar, nonpolar etc.), information about the chemical requirements at each position for mAb recognition can be obtained. Moreover, the positions critical for recognition are

also determined. Thus, a complete set of substitution analogs for the 14-mer peptide isolated by SA-3 would contain 280 peptides, and for the 12-mer isolated by SYA/J6 would contain 240. (These numbers include fourteen or twelve copies, respectively, of the parent peptide sequences as redundant controls.)

Due to cost considerations, we wanted to reduce the number of analogs synthesized. Since we already knew the residues important for mAb binding, we decided to replace these residues only because we were interested in determining how strict the requirements were for the chemical functionalities presented by the critical binding residues. To reduce the number of analogs further, the twenty amino acids were arranged into eight groups according to similarities between their side chains and one or two amino acids from each group were chosen for the substitutions. As a result, all the substitution analogs were synthesized on the same block as the peptides synthesized to determine the minimum length required.

The grouping of amino acids and the amino acids chosen to replace a specific amino acid in the parent sequences of the SA-3 isolated peptide and the SYA/J6-isolated peptide are shown in Table 4.7. The largest group of amino acids included alanine, serine, glycine, threonine, cysteine and methionine. These amino acids were grouped together because they all have small side chains. Furthermore, alanine, glycine and serine are self-replacing, as previously mentioned, and serine and threonine both have a polar hydroxyl group that is similar to the sulfhydryl group of cysteine. Two other groups of amino acids had three members each; isoleucine, leucine and valine were grouped together, as were phenylalanine, tyrosine and tryptophan. Isoleucine, leucine, and valine

all have small, nonpolar side chains whereas phenylalanine, tyrosine and tryptophan all have large aromatic side chains. Arginine and lysine were grouped together since they both have positively charged, basic side chains. Aspartic acid and glutamic acid were grouped together, as were asparagine and glutamine, since they have negatively charged, acidic side chains or polar, amide-containing side chains, respectively. Finally, proline and histidine both formed their own groups.

Table 4.7. Amino acid groups and choice of amino acids for substitution analogs

	Amino acid chosen for replacement of								
	SA-3-isolated peptide				SYA/J6-isolated peptide				
Amino acid group	G	A	R	P	M	W	N	A	A
A, S, G, T, C, M	T	T	T	T	T	T	A, T	T	T
I, L, V	I	I	I	L	I	L	L	I	I
F, Y, W	Y	Y	Y	F	F	F	Y	Y	Y
R, K	K	R, K	K	R	K	R, K	-	R, K	R, K
D, E	D	D	E	D	E	E	D	D	D
N, Q	N	N	Q	N	Q	Q	Q	N	N
P	P	P	P	-	P	P	P	P	P
H	H	H	H	H	H	H	H	-	H

For the SA-3-isolated peptide, the first glycine, the second alanine, the arginine and the first proline were substituted (ADGADRPVPYGACG). The chemical requirements at the final position were not investigated since it was difficult to believe this residue was critical for binding in the context of the originally-selected sequence; even though the data in Table 4.3 suggested the final glycine was important, it was not part of the originally-selected sequence (Chapter 3).

Table 4.7 shows the amino acids used for the different substitution analogs of the SA-3-isolated peptide sequence. In general, threonine was chosen since substitution by

serine or alanine would not have provided much information due to their close similarity to glycine. Cysteine and methionine were never chosen due to synthetic complications related to their sulfur containing side chains; the formation of any potential disulfide bonds is difficult to control and methionine is easily oxidized. Tyrosine was chosen since tyrosine substitution would complement threonine substitution; both side chains have hydroxyls but their sizes are very different. Therefore, together, the data for the threonine-and tyrosine-substitution analogs may give an indication of the size of the side chain allowed. It would be difficult to determine if a phenylalanine- or a tryptophan-substitution analog was not bound due to the large side chain or due to the lack of hydrogen bonding. However, phenylalanine was chosen to replace the proline residue in the parent sequence in order to probe the size requirements only at this position since phenylalanine is incapable of hydrogen bonding. The other amino acids used for the substitution analogs were chosen based upon the length of their side chains.

As can be seen in Table 4.8, only the lysine-substitution analog of the first glycine position showed any loss of activity. Even the tyrosine-substitution analog was active, contrary to the datum in Table 4.3 which shows some activity. The data in Table 4.3 was obtained by testing only one substitution analog for each residue, while the data in Table 4.8 was obtained by testing eight different analogs of the first glycine. Therefore, the data in Table 4.8 should be more reliable and indicate that the first glycine is not critical for SA-3 binding, as may have been expected for a residue from the constant cpIII region (60). By the same token, the decrease in activity observed for the lysine-substitution

analog may not be reproducible; however, it may be that the positive charge of lysine's side chain does disrupt SA-3 recognition of this peptide.

The data in Table 4.8 illustrate that all the substitution analogs of the first alanine were active. Even the tyrosine-substitution analog was just as active as the parent peptide, contrary to the datum in Table 4.3 which showed a significant decrease in activity. Once again, since the data in Table 4.8 were obtained by testing nine different analogs, compared to the one substitution analog tested in Table 4.3, the data in Table 4.8 should be more reliable and indicate that this alanine is not critical for SA-3 binding, as may have been expected for a residue from the constant cpIII region (60).

Table 4.8. SA-3 binding to substitution analogs of the peptide corresponding to the SA-3-isolated sequence.

Amino acid substituted	Peptide sequence	$(A_{405}-A_{490}) \times 1000$
	ADGADRPVPYGACG	1615
	AD**H**ADRPVPYGACG	1254
	AD**K**ADRPVPYGACG	**338**[a]
	AD**P**ADRPVPYGACG	1698
G	AD**I**ADRPVPYGACG	1204
	AD**T**ADRPVPYGACG	1499
	AD**Y**ADRPVPYGACG	1258
	AD**N**ADRPVPYGACG	1744
	AD**D**ADRPVPYGACG	1796
	ADG**H**DRPVPYGACG	1332
	ADG**K**DRPVPYGACG	1689
	ADG**P**DRPVPYGACG	1666
A	ADG**I**DRPVPYGACG	1509
	ADG**T**DRPVPYGACG	1555
	ADG**Y**DRPVPYGACG	1233
	ADG**N**DRPVPYGACG	1571
	ADG**D**DRPVPYGACG	1272
	ADG**R**DRPVPYGACG	1262
	ADGAD**H**PVPYGACG	**93**
	ADGAD**K**PVPYGACG	**66**
	ADGAD**P**PVPYGACG	**92**
R	ADGAD**I**PVPYGACG	**97**
	ADGAD**T**PVPYGACG	**92**
	ADGAD**Y**PVPYGACG	**111**
	ADGAD**Q**PVPYGACG	**139**
	ADGAD**E**PVPYGACG	**147**
	ADGADR**H**VPYGACG	**73**
	ADGADR**L**VPYGACG	**121**
	ADGADR**T**VPYGACG	**76**
P	ADGADR**F**VPYGACG	**94**
	ADGADR**N**VPYGACG	**106**
	ADGADR**D**VPYGACG	**56**
	ADGADR**R**VPYGACG	**85**
	background	55

a) numbers in bold indicate a significant decrease in SA-3 binding as compared to SA-3 binding to the parent peptide sequence.

The data in Table 4.8 demonstrates that none of the substitution analogs of the arginine or proline were active. These data not only support the data in Table 4.3, but also indicate that the requirement for arginine and proline at these position are very strict. The strict requirement for proline is not surprising given its unique structure.

In summary, the data in Table 4.8 obtained from the set of substitution analogs based on the SA-3-isolated peptide, along with the data obtained from the alanine substitution experiments (Table 4.3) and the amino acid deletion studies (Table 4.5), suggest that the only residues critical for binding are the arginine and proline (ADGAD**RP**VPYGACG); furthermore, only arginine and proline fulfill the chemical requirements for binding at these positions. This result is similar to the result of the alanine-substitution (Table 4.3), which suggests that the first glycine and second alanine are not as critical for SA-3 recognition as the arginine and proline, and supports at least part of the consensus sequence **RP**XXY determined from biopanning (Table 3.2).

For the SYA/J6-isolated peptide, the first methionine, the tryptophan, the asparagine and the two alanines were substituted (MDWNMHAAEGDD). Table 4.7 shows the amino acids used for the different substitution analogs of the SYA/J6-isolated peptide sequence. In general, the other amino acids used for the substitution analogs were chosen based upon the length or size of their side chains. For the tryptophan-substitution analogs, phenylalanine was chosen rather than tyrosine, to investigate the chemical requirements for a large hydrophobic side chain, since its side chain is large and hydrophobic like tryptophan's but incapable of forming hydrogen bonds like tyrosine. However, tyrosine was chosen for the asparagine-substitution analogs due to its hydrogen

bonding abilities; the tyrosine-substitution analog would provide insight into the size requirements for a hydrogen-bonding side chain at this position. Furthermore, aspartic acid was chosen since it is the acidic counterpart to asparagine and glutamine was chosen in order to probe the size requirement at this position. Since the chemical requirements at the second alanine appeared to be more flexible, the histidine-substitution analog was included for this position.

As can be seen in Table 4.9, only the lysine-and histidine-substitution analogs of the methionine lost activity; moreover, the isoleucine-and threonine- substitution analogs were significantly more active than the parent peptide. These data are consistent with the data in Table 4.4; all suggest that the first methionine plays a role in SYA/J6 recognition but the chemical requirements for the methionine side chain are not strict at this position. In fact, only basic groups do not seem to be well tolerated in this position.

The data in Table 4.9 shows that only the phenylalanine-substitution analog of the tryptophan position had activity above background, and none of the substitution analogs of the asparagine position were active. Therefore, these data, and those in Table 4.4, suggest that the tryptophan asparagine are a critical binding residues. Moreover, the data in Table 4.9 indicate that the chemical requirements, at the tryptophan position, for a large, hydrophobic side chain are quite strict; as are the chemical requirements, at the asparagine position, for an amide group one carbon away from the amino acid α-carbon.

Table 4.9. SYA/J6 binding to substitution analogs of the peptide corresponding to the SYA/J6-isolated sequence.

Amino acid substituted	Peptide sequence	$(A_{405}-A_{490}) \times 1000$
	MDWNMHAAEGDD	754
	KDWNMHAAEGDD	**255**[a]
	PDWNMHAAEGDD	972
	IDWNMHAAEGDD	1130
M	**T**DWNMHAAEGDD	1278
	FDWNMHAAEGDD	836
	QDWNMHAAEGDD	965
	EDWNMHAAEGDD	911
	HDWNMHAAEGDD	498
	MD**H**NMHAAEGDD	**101**
	MD**P**NMHAAEGDD	**29**
	MD**L**NMHAAEGDD	**44**
	MD**T**NMHAAEGDD	**37**
W	MD**F**NMHAAEGDD	**236**
	MD**Q**NMHAAEGDD	**30**
	MD**E**NMHAAEGDD	**33**
	MD**R**NMHAAEGDD	**32**
	MD**K**NMHAAEGDD	**37**
	MDW**P**MHAAEGDD	**42**
	MDW**Y**MHAAEGDD	**56**
	MDW**A**MHAAEGDD	**58**
N	MDW**T**MHAAEGDD	**44**
	MDW**L**MHAAEGDD	**29**
	MDW**Q**MHAAEGDD	**28**
	MDW**D**MHAAEGDD	**34**
	MDW**H**MHAAEGDD	**47**
	MDWNMH**K**AEGDD	**34**
	MDWNMH**P**AEGDD	**39**
	MDWNMH**I**AEGDD	**153**
A	MDWNMH**T**AEGDD	**90**
	MDWNMH**Y**AEGDD	**40**
	MDWNMH**N**AEGDD	**37**
	MDWNMH**D**AEGDD	**46**
	MDWNMH**R**AEGDD	**30**

Table 4.9 (concl'd). SYA/J6 binding to substitution analogs of the peptide corresponding to the SYA/J6-isolated sequence.

Amino acid substituted	Peptide sequence	$(A_{405}-A_{490}) \times 1000$
	MDWNMHAHEGDD	**107**
	MDWNMHAKEGDD	**88**
	MDWNMHAPEGDD	**36**
	MDWNMHAIEGDD	**135**
A	MDWNMHATEGDD	**238**
	MDWNMHAYEGDD	**340**
	MDWNMHANEGDD	**193**
	MDWNMHADEGDD	**337**
	MDWNMHAREGDD	**65**
	background	55

a) numbers in bold indicate a significant decrease in SA-3 binding as compared to SA-3 binding to the parent peptide sequence.

Different substitution analogs were active for the two different alanine positions (Table 4.9). Only the isoleucine-substitution analog of the first alanine had any activity above background levels; whereas the isoleucine-, threonine-, tyrosine-, asparagine-, and aspartic acid- substitution analogs of the second alanine all had activity above background levels. Thus, these data, and those in Table 4.4, suggest that both alanines are critical binding residue. However, the data in Table 4.9 suggest that the requirements for a small hydrophobic side chain are much more strict at the first alanine position than at the second. Small hydrophobic side chains and side chains capable of hydrogen bonding, regardless of size, can all be recognized at the second alanine position. The conclusion that the two alanines, from the flanking region, are critical binding residues was not expected from the biopanning experiments. All of the sequences selected in biopanning experiments contained the same amino acids in the flanking region; therefore, SYA/J6 did not select these amino acids. Hence, the two flanking region alanines may be

required for producing the correct conformation rather than for forming critical contacts with SAY/J6.

In summary, the data in Table 4.9 obtained from the set of substitution analogs based on the SYA/J6-isolated sequence, along with the data obtained from the alanine substitution experiments (Table 4.4) and the amino acid deletion studies (Table 4.6), suggest that the very first residue is important for recognition but the requirement for methionine is not strict (**MDWNMHAAEGDD**). Furthermore, tryptophan and asparagine are critical residues for binding and the requirement for these amino acids is quite strict. Finally, the two flanking region alanines are both important for recognition, but the requirement for alanine is more strict in the first position than in the second. This result is similar to the result of the alanine-substitution (Table 4.4), which suggests that the first methionine and second alanine in the flanking region are not as critical for SYA/J6 recognition as the tryptophan, asparagine and first alanine in the flanking region. Neither result from the alanine-substitution nor the larger substitution study support the consensus sequence **NM** determined from biopanning.

4.1.6 Synthesis of the SYA/J6-based sequence, MDWNMHAA, to study further its interaction with SYA/J6.

No crystal structures of the anti-dGAS mAbs exist. Thus, while some of the anti-dGAS mAbs-isolated peptides were recognized when synthesized on a pin, structural studies of these mAb:peptide complexes and comparisons with the mAb:carbohydrate complexes would be limited. In contrast, there are x-ray and microcalorimetry data for SYA/J6 complexes with carbohydrates (98, 100). Thus, a SYA/J6-isolated peptide was

selected for further study. The sequence MDWNMHAA was chosen since it was the shortest sequence recognized; moreover, its activity was actually greater than that of the parent peptide (Table 4.6). A short sequence was preferable because it would be less expensive to have commercially synthesized and any data obtained for the mAb:peptide complex should be easier to interpret. No amino acids were changed from the parent peptide even though the data in Table 4.9 suggest that activity may be increased by substituting the first methionine with isoleucine or threonine. The isoleucine-and threonine-substitution analogs studied contained all the amino acids of the parent peptide; shortening the sequence could affect the chemical requirements for binding. Thus, it could not be assumed that substitution analogs of a shortened peptide would behave similarly to those of a longer peptide.

In order to determine if SYA/J6 recognized the MDWNMHAA-peptide free in solution, the peptide was used to inhibit SYA/J6 binding to its LPS. The data in Table 4.10 show that the MDWNMHAA-peptide did inhibit SYA/J6 binding to its immobilized LPS; therefore, the soluble MDWNMHAA-peptide was recognized by SYA/J6 at or near the carbohydrate binding site. The multivalent nature of the immobilized antigen affects the behavior of the bivalent Ab. In order to inhibit Ab binding to the LPS, enough peptide must present to completely interact with one of the Ab combining sites. As a result, the inhibitor is not well-behaved at low concentrations, which explains the apparent increase in inhibition for 0.5 μM peptide compared to 5 μM peptide.

Table 4.10. Percent inhibition by MDWNMHAA-peptide of SYA/J6 binding to LPS

Peptide concentration	Percent inhibition
50 μM	100%
5 μM	44%
0.5 μM	51%

Dr. David Bundle at the University of Alberta used titration microcalorimetry to study the thermodynamics of the interaction between SYA/J6 and the MDWNMHAA-peptide. The titration curve shown in Figure 4.1 for the interaction of SYA/J6 with the MDWNMHAA-peptide was well behaved. The data in Table 4.11 show that the affinity of the MDWNMHAA-peptide is greater than that of the native trisaccharide (**BCD**, see Figure 2.5), but less than that of a deoxy-modified trisaccharide (98). The enthalpy of binding of the MDWNMHAA-peptide:SYA/J6 interaction is favorable and similar to that of the native trisaccharide (**BCD**); however, the entropy of binding of the MDWNMHAA-peptide:SYA/J6 interaction offsets the favorable enthalpy. As a result, the MDWNMHAA-peptide's affinity is not as great as that of a deoxy-modified trisaccharide (98). Dr. F. Quiocho of the Howard Hughes Medical Institute is attempting to obtain a crystal for the Fab fragment of SYA/J6 complexed with the MDWNMHAA-peptide.

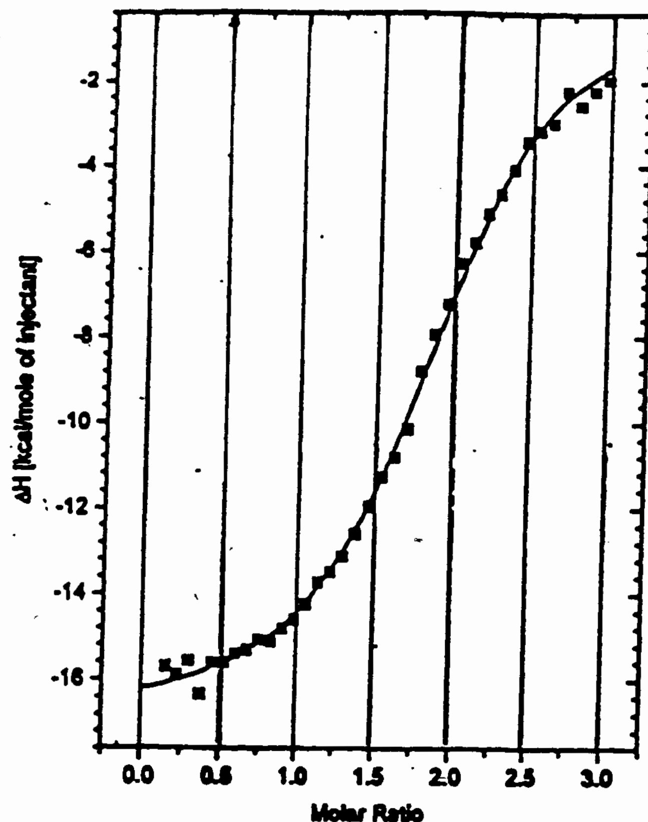


Figure 4.1. The titration curve for SYA/J6 binding to the MDWNMHAA-peptide.

Table 4.11. Thermodynamic parameters of SYA/J6 binding to the MDWNMHAA-peptide and two carbohydrates

	MDWNMHAA-peptide	Native trisaccharide (**BCD**) (98)	A monodeoxy trisaccharide (**BC'D**) (98)
K_a (M^{-1})	(5.7 ± 0.3) x 10^5	(9.2 ± 1.3) x 10^4	(6.4 ± 3.0) x 10^6
ΔG (kcal/mol)	-7.85 ± 0.05	-6.77 ± 0.07	-9.3 ± 0.4
ΔH (kcal/mol)	-16.9 ± 0.8	-4.7 ± 0.2	-9.6 ± 0.1
TΔS (kcal/mol)	-9.04 ± 0.9	2.1 ± 0.2	-0.3 ± 0.3

4.2 Discussion

The peptide sequences investigated in Chapter 3 were synthesized on pins using the Multipin™ peptide synthesis kit from Chiron Mimotopes (31-34). In general, the cross-reactive peptides were recognized, above the background level of a blank pin, by the anti-carbohydrate mAb used for their isolation bound, but the specificity

of the mAbs for their corresponding peptides was low (Table 4.1). Moreover, the peptides corresponding to sequences isolated by Strep 9, HGAC 101 or Se155.4 were not recognized by their corresponding mAb above background levels. It is unclear why the Strep 9-isolated peptides were not bound by Strep 9. It is possible that the disulfide bonds required for Strep 9 recognition (Table 3.11) were not formed correctly in the peptides synthesized on the pins. Furthermore, cpVIII (or at least a few of the first residues) may be required for the formation of the necessary conformation. The affinity of HGAC 101 and Se155.4 for its isolated peptides were low when they were displayed on phage (Table 3.2). Thus, the affinity of HGAC 101 or Se155.4 binding to their corresponding peptides on pins may not have been strong enough to be detected, even with a potential increase in avidity due to the increased density of peptides displayed on the pins (32, 33, 60, 66).

The specificity of the mAbs for their corresponding peptide synthesized on a pin was low and the patterns of reactivity were not the same as when the peptides were displayed on phage (Table 4.1). For instance, even though the Strep 9-isolated sequence MCPPLYSPSAC was not bound by Strep 9, it was bound by all the other anti-dGAS mAbs. In comparison, only Strep 9 and SA-3 bound the peptide when it was displayed on phage (Table 3.5). One HGAC 39-isolated peptide synthesized on pins was bound by both HGAC 39 and SA-3 (Table 4.1); however the specificity of this peptide was not tested when it was displayed on phage. The other HGAC 39-isolated peptide was bound by HGAC 39 alone when synthesized on a pin (Table 4.1), but was bound by both HGAC 39 and SA-3 when displayed on phage (Table 3.5). The one HGAC 47-isolated peptide,

bound by HGAC 47 above background levels, was specific for HGAC 47; thus, this peptides was as specific as when displayed on phage (Table 3.5).

It is not clear whether the pin-display of cross-reactive peptides can be used to probe the interaction of phage-displayed peptides with mAbs. The activity, specificity and general pattern of reactivity with the mAbs used for this work was different for the two modes of peptide display (Table 4.1); in general, this method worked for only two of the five anti-dGAS mAbs. The reasons for the different activity patterns are unclear; it may be due to the synthetic peptides themselves, such as lack of correct disulfide formation, problems arising in the synthesis that were not detected and/or the density of the peptides on the pins (32, 33, 60, 66). The ability of all the anti-dGAS mAbs, except Strep 9, to bind the Strep 9-isolated sequence MCPPLYSPSACA suggests that the avidity of the interactions may be increased; however, the avidity of the interactions between HGAC 101 and Se155.4 and their respective peptides either did not increase, or did not increase enough, for binding to be observed. The solid support provided by the pins is very different from the protein environment provided by the coat proteins for the phage-displayed peptides. The large coat protein may help "solubilize" the small peptide. The coat protein probably does not induce a particular conformation in the peptide, since in other studies performed in the Scott laboratory, peptides can routinely be moved from the *N*-terminus of cpVIII to the *N*-terminus of maltose-binding protein of *E. coli* without affecting activity (11 of 12 examples tested).

The positions critical for binding and the chemical requirements at these positions for mAb recognition were determined for two sequences regardless of any

limitations. The activity of SA-3 and SYA/J6 for peptides on pins was similar to that for phage-displayed peptides; thus, a SA-3- and a SYA/J6-isolated peptide were chosen for further study. Moreover, the DRPVPY-peptide was chosen because it was our most studied peptide (Chapter 3, ref. 29), and there are a great deal of data for SYA/J6:carbohydrate interactions to which similar data for SYA/J6:peptide interactions could be compared (98, 100).

All of the peptides were not studied in more detail due to the cost of synthesizing several large sets of peptides with the Multipin™ kit. The major chemical cost for a synthesis is the solvents used for the wash baths after Fmoc deprotection and coupling of amino acids. All the pins are washed together for these common steps; thus, large quantities are used. However, the quantity of solvent used for the wash baths is the same whether one pin or ninety-six pins are used in a block. (Fresh wash baths are needed for each block.) Therefore, the most efficient use of the solvents is to synthesize full blocks of pins. Furthermore, the labor costs need to be considered; an organized researcher can couple three amino acids in one ten hour day. Thus, 15-mer peptides can be synthesized in five long work days. Consequently, the syntheses performed for this work were designed to completely fill only one block at a time; therefore, only two peptides were studied in depth and only a limited number of substitution analogs were studied for these peptides.

A study of alanine-substitution analogs illustrated that the arginine and the first proline of the DRPVPY-peptide (ADGADRPVPYGACG) were critical for SA-3 binding (Table 4.3). Moreover, the first glycine and second alanine from the cpIII portion of the

peptide, as well as the final glycine from the "linker-arm" portion of the synthetic DRPVPY-peptide, were affected by alanine substitution. These results confirm the importance of arginine and proline in the consensus sequence **RPXXY** suggested by the sequences isolated by biopanning (Chapter 3, ref. 29). It is not possible to deduce a role for residues within constant regions by sequence comparison only; thus, the use of alanine-scanning to determine the critical residues for binding complements the determination of a consensus sequence from biopanning experiments. However, the residues determined to be critical for binding using the peptides on pins technology may not be the same residues critical for binding of phage-displayed peptides, or even of peptides in solution, since the behavior of the DRPVPY-peptide on the pin was not the same as on the phage, or in solution (Table 4.1).

The Multipin™ technology was also used to determine the chemical binding requirements at the critical positions (31, 33, 35). Several different substitution analogs for each of the critical positions were synthesized; the chemical requirements were determined by determining which analogs were recognized by the mAb used to isolate the parent sequence. Hence, it was determined that only arginine and proline are able to fulfill the chemical requirements of the two critical binding positions of the DRPVPY-peptide (Table 4.8). This result was not too surprising given the length and functionality of arginine's side chain and the unique nature of proline. The other positions shown to be critical for binding by the alanine-substitution study were not affected by the different amino acid substitutions used for this more detailed study. Thus, it appears that only

arginine and the first proline are strictly required for SA-3 recognition of the DRPVPY-peptide when displayed on a pin.

The SYA/J6-isolated sequence MDWNMHAAEGDD was also investigated to determine the critical residues for binding and the chemical requirements at these positions for SYA/J6 recognition. The alanine-substitution study illustrated that the tryptophan and the asparagine, along with the first alanine, were critical for SYA/J6 binding (Table 4.4). Moreover, the first methionine and the second alanine played a role in the peptide's activity since SYA/J6 binding was affected by their substitution with alanine (Table 4.4).

These results confirmed the importance of asparagine in the consensus sequence **NM**, suggested from biopanning experiments (Chapter 3, ref. 29). However, the second methionine was not shown to be important for binding, whereas several other residues were shown to be important. The importance of the first methionine and the tryptophan may have been difficult to deduce from the biopanning experiments since MDWNMHAAEGDD was the only tight-binding sequence isolated from the X_6 library (Table 3.2). The consensus sequence **NM** was deduced by comparing this X_6 sequence to sequences isolated from the $X_4CX_4CX_4$ library; the recognition rules of the $X_4CX_4CX_4$ library may be different from those of the X_6 library. Furthermore, the importance of either alanine to this sequence could not be deduced from sequence comparisons since these residues were within the constant cpVIII regions (30). Therefore, for this SYA/J6-isolated peptide, it appears that alanine-scanning gave a much clearer indication of the critical binding residues than did the consensus sequence. Moreover, the activity of the

peptide on the pin was similar to that on the phage (Table 4.1); thus, it should be possible to relate the findings for the pin-display of this peptide to the phage-displayed peptide.

More substitution analogs of the critical positions of the SYA/J6-isolated peptide, MDWNMHAAEGDD, were investigated in order to determine the chemical requirements at these positions. The chemical requirements for SYA/J6 recognition were quite strict at some positions, but more flexible at others (Table 4.9). Only tryptophan and asparagine were able to fulfill the chemical requirements at their positions, respectively. The phenylalanine replacement of the tryptophan did give a peptide that exhibited some activity, suggesting that the requirements at this position are for a large, hydrophobic side chain. Neither the aspartic acid- nor the glutamine-substitution of the asparagine gave peptides that were active, suggesting that the requirement for an uncharged side chain, two carbons long is very strict at this position. None of the substitution analogs of the first alanine position were active, suggesting a strict requirement for a small side chain. The chemical requirements at the other two positions shown to be important for SYA/J6 binding were not as strict. All of the substitution analogs of the first amino acid were active; only lysine- and histidine-substitution showed any significant loss of activity. It appears, therefore, that any non-basic side chain can be accommodated in the first position. An arginine-substitution analog of the first methionine position was not synthesized; an arginine-substitution analog may have provided more support for the observation that basic side chains in the first position were not active. Only the arginine, histidine, lysine and proline analogs of the second alanine position were not active, suggesting that this position, too, will accept any non-basic side

chain. Since proline is a unique amino acid, it was not surprising that the proline-substitution analog was not active.

The study of many different substitution analogs demonstrates how the Multipin™ technology can be used to complement biopanning experiments. Comparisons of the sequences isolated from biopanning experiments provide an indication of the chemical requirements for binding at a position (29, 30, 60). However, in general, only a small number of clones were analyzed in our biopanning experiments. It is difficult to deduce if a closely related sequence was not isolated because strict chemical requirements were not fulfilled or because they were just lower affinity sequences which were overwhelmed during the biopanning experiments (30, 60). Furthermore, sequences that do not bind are rare in pools that are enriched for binding phage; therefore, it is impossible to show directly that an amino acid does not fulfill the chemical binding requirements.

In order to control costs, a two-step process was used in this thesis to investigate the chemical requirements of peptide binding for two anti-carbohydrate mAbs. First one substitution analog of each amino acid was tested to determine which positions to investigate. If this one analog exhibited decreased activity, the chemical requirements of the corresponding position were further investigated. In the case of the DRPVPY-peptide, analysis of a single substitution analog suggested that residues from the constant cpIII region were important for recognition. However, when these positions were probed with more substitution analogs, no role was suggested. Thus, this two-step approach provided a more in depth double check of the initial results. However, just as the single

substitution analog suggested a role for a position that was not supported by more in depth probing, a critical residue may have been missed by the initial screen. If the cost of a large synthesis is not an issue, analysis of a complete set of substitution analogs, or at least a large number at every position, would more reliably indicate the important residues and, at the same time, the chemical requirements for binding.

A phage-displayed peptide library constructed such that the conserved positions were held constant while the other positions were allowed to vary could be used to search for sequences with greater activity. However, the effect of all replacements, or particular replacements, will not be determined since there is no choice as to what sequence is selected by biopanning; selection is a random draw from a pool of sequences covering a range of affinities. The Multipin™ technology provides a direct method for testing specific analogs of a consensus sequence. Thus, if the behavior of the peptide is similar when on the pin as when displayed on a phage, the chemical requirements for activity at any given position can be investigated (31, 33, 35). Once the critical residues and the chemical requirements for binding at those positions had been determined, it would be necessary to test the various amino acid combinations. The critical residues could be held constant and the remaining positions could be replaced by all other amino acids; thus, the sequence of an active peptide with favorable properties such as solubility and little flexibility could be determined. The peptides synthesized for such optimization experiments could be synthesized using the Multipin™ cleavable peptide kit. Peptides made using the cleavable kit can be removed from the pin support used for their synthesis. Thus, the fidelity of the sequences can be confirmed, the peptides can be used

directly in more biologically relevant solution assays, and any effect of the coupling to a solid support is removed.

Shortened analogs of the SA-3-isolated peptide and the SYA/J6-isolated peptide were studied. It is more practical to study the interactions of shorter peptide sequence since they are less expensive to synthesize and there are fewer amino acids to consider when interpreting data. Thus, in order to determine the minimum length required for SA-3 or SYA/J6 recognition, peptides corresponding to shortened portions of the parent peptide sequences were studied. Unfortunately, due to cost considerations, the synthesis of these peptides was performed along with the larger substitution study. It may have been more productive to have determined the minimum length required before the large substitution study and then determined the critical residues and chemical requirements for the shortened peptides. Nonetheless, the data in Table 4.5 and Table 4.6 suggest that an octapeptide was the minimum length bound by either SA-3 or SYA/J6. In the case of SA-3, a heptapeptide may also be recognized, but such a peptide was not tested. For SYA/J6, not only was an octapeptide the shortest peptide bound, it was also bound more tightly than the parent sequence.

One of the goals of the analog studies was to determine which peptides to synthesize as free molecules. Soluble peptides could be used to study mAb:peptide complexes in greater detail. For instance, peptides could be co-crystallized with mAbs, used in TRNOE studies or in titration microcalorimetry experiments. A good peptide candidate would be short, hydrophilic, and have a high affinity for the mAb. Based on the analogs studied, including some shortened versions of the parent sequences, one

sequence was chosen. Due to the wealth of information collected for SYA/J6:carbohydrate complexes, (98, 100) the sequence MDWNMHAA, a shortened version of the SYA/J6-isolated peptide, was synthesized. No amino acid substitutions were made to this shortened sequence, since all the substitution analogs studied were synthesized within the context of the entire sequence, the effect that substitutions would have upon the activity of the shortened peptide was not obvious.

Titration microcalorimetry studies of the SYA/J6:MDWNMHAA-peptide interaction indicated that whereas the MDWNMHAA-peptide's affinity for SYA/J6 is greater than that of the native trisaccharide (**BCD**), it is not as great as that of a deoxy-modified trisaccharide (98). In contrast, the DRPVPY-peptide's affinity for SA-3 was greater than the best-binding carbohydrate. The enthalpy of binding for the MDWNMHAA-peptide was favorable, however, it was offset by the entropy of binding. Decreasing the flexibility of the unbound peptide would decrease the change of entropy upon complex formation may increase the affinity of the MDWNMHAA-peptide for SYA/J6.

The MDWNMHAA-peptide is being co-crystallized with the Fab fragment of SYA/J6. The peptide amino acids shown to interact with the mAb in the crystal structure could be compared to those amino acids shown to be critical by the various substitution analogs. Moreover, the surface presented by the bound peptide could be compared to the surface presented by bound carbohydrates; the amino acids used by mAb binding site to bind either carbohydrates or peptides could also be compared. Comparisons of the SYA/J6:peptide complex to the SYJ/J6:carbohydrate complexes should provide a great

deal of information about the mechanisms of binding of the different ligands. The crystal structure data for the SYA/J6:peptide complex could also be used to direct further peptide analog studies. The crystal structure would provide direct evidence of which amino acids interact with SYA/J6 and the size and functionalities that could be accommodated at other positions as well as optimal constraints on the peptide's flexibility.

4.3 Materials and Methods

The peptide, NH_2MDWNMHAA-$CONH_2$ (MDWNMHAA-peptide), was synthesized and HPLC-purified by the Alberta Peptide Institute (Edmonton, Alta.).

The Multipin™ kits used for the synthesis of peptides on pins were purchased from Chiron Mimotopes (San Diego CA). The kits included the derivatized pins, blocks for holding the pins during the synthesis, solvent-resistant wash baths and the software needed to generate the synthetic schedules. The protected amino acids, diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were purchased from Bachem (Torrence, CA). The solvents used were purchased from Sigma (St. Louis, MO) and were distilled before use.

4.3.1 Synthesis of peptides on pins (31-35)

Fmoc-deprotection and washing. Pins were placed into position within a 8 x 12 block. The pins never changed their position within the block, thus the peptide sequence was determined by the amino acids added at a given position. The first step in the peptide synthesis was Fmoc-deprotection of the growing *N*-terminus. The block of pins

was placed in a bath containing piperidine (20% in dimethylformamide (DMF)) for 20 min at room temperature. The bath contained enough piperidine solution to cover the ends supporting the growing peptides. After the Fmoc-deprotection, the pins were washed by immersing the pins in a bath of DMF for 2 min, followed by immersion in three baths of methanol for 2 min each. The pins were allowed to air dry for 30 min after the washings.

Coupling and deprotection of N-α-Fmoc-protected amino acids. Preparation of amino acid solutions. The amino acid solutions used for the coupling procedures were made in distilled DMF. The software package purchased from Chiron Mimotopes was used to generate tables of the required quantities of all the reagents needed for each coupling cycle. The coupling solutions contained 100 mM of each amino acid, 100 mM of DIC and 120 mM of HOBt. Stock solutions of DIC and HOBt were prepared for each coupling cycle. Each amino acid was first dissolved in an aliquot of the HOBt solution and then an aliquot of the DIC solution was added.

Dispensing activated solutions, amino acid coupling and side-chain deprotection. Immediately after activation, 150 μL of the activated amino acid solutions were dispensed into the appropriate position of a 96-well reaction tray. After all the amino acids had been dispensed, the block of pins was placed, with the correct orientation, into the 96-well reaction tray. The block of pins and reaction tray were placed in a sealed container to minimize evaporation during the coupling reaction. The coupling reaction was allowed to proceed at room temperature for 2h or overnight.

If the next coupling cycle was to proceed immediately, the pins were immersed in a methanol bath and agitated for 5 min, air dried for 2 min and immersed in a DMF bath for 5 min. The pins were then ready for the next cycle beginning with Fmoc-deprotection. If the next coupling cycle was not to proceed immediately, the pins were immersed in a DMF bath and agitated for 5 min, immersed two times in methanol baths for 2 min each and allowed to air dry. The block of pins was then stored dry in a clean plastic bag at room temperature until needed.

The deprotection, washing, coupling and washing steps were repeated until the peptide sequences were complete. When complete, the terminal Fmoc group was removed as previously described and then the side-chains were deprotected. Side-chain deprotection was accomplished by immersing the pins in a mixture of trifluoroacetic acid:ethanedithiol:anisole (38:1:1) for 2.5 h at room temperature. The pins were first immersed in methanol for 10 min after the side-chain deprotection. Next the pins were immersed in a methanol:water (1:1) solution containing acetic acid (5%) for 60 min. Finally, the pins were washed two times in methanol baths for 2 min each. The pins were ready for testing after air drying overnight.

4.3.2 ELISA for peptides on pins (31)

The pins were blocked with blotto (5% milk powder in TBS (50 mM TrisHCL, 150 nM NaCl, pH 7.5)) in order to reduce non-specific binding. Blotto (200 μL) was added to microwells and the pins were placed in the wells for 60 min at room temperature. The pins were washed by immersion into TBS for 10 min. The pins were

then placed in wells containing the primary Ab solution (200 μL of 100 nM Ab in blotto) and incubated for 4 h at room temperature. The pins were washed four times by immersion into baths of TBS for 10 min each time. To detect bound primary Ab, the pins were placed in wells containing a secondary Ab conjugated to horseradish peroxidase for 60 min at room temperature. The pins were washed four times by immersion into baths of TBS for 10 min each time and then placed in wells containing a solution of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonate) (200 μL). The reactions were stopped by simply removing the pins. Optical densities are reported as (A_{405}-A_{490}) x 1000.

The pins could be reused by removing the bound Abs. The pins were placed in a sonication bath with a TBS solution containing sodium dodecyl sulfate (SDS, 1%) and 2-mercaptoethanol (0.1%). The temperature of the solution was between 55°C and 65°C and the pins were sonicated for 10 to 20 min. The pins were rinsed twice with water at 60°C for 30 sec each time and then placed in a water bath and agitated for 30 min. For the final rinse with water, the pins were placed in a water bath at 60°C which was left to cool at room temperature while the pins were agitated. Finally, the pins were rinsed with methanol for 1 min and left to air dry.

The test for the successful removal of the Abs was to place the pins in wells containing the secondary Ab conjugated to horseradish peroxidase, washing the pins and then placing them in wells containing 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonate) solution. If the solution remained uncolored, the removal of Abs was found to be successful. If the removal was not successful, the sonication was repeated.

4.3.3 Competition ELISA for the MDWNMHAA-peptide

Microwells were coated overnight at 4°C with the LPS from *Shigella flexneri* Y (40μL of 1 μg LPS/mL TBS) or with blotto. The wells were washed three times with TBS containing Tween 20 (0.5%, TBS/Tween) and blocked with blotto (200 μL) for 20 min at 37°C. The wells were washed three times with TBS/Tween. Wells received equal volumes of SYA/J6 solution and peptide solution; control wells received blotto rather than peptide solution. The final concentration of SYA/J6 was 60 nM; the final concentrations of the peptide were 50 μM, 5 μM and 0.5 μM. After incubating overnight at 4°C, the wells were washed six times with TBS/Tween and bound Ab was detected with a secondary Ab conjugated to horseradish peroxidase. Optical densities are reported as $(A_{405}-A_{490})$ x 1000.

CHAPTER 5

CHARACTERIZATION OF GLYCOAFFINITY REAGENTS

5.1 Introduction

Affinity reagents consisting of an immobilized ligand on a solid support have become very popular for both analytical and preparative purposes since mild conditions are used to obtain very pure products in high yields (127). Affinity reagents can be used to isolate macromolecules present in low concentration within complex mixtures from various sources; thus, they are ideal for the purification of Abs from sera. Unlike precipitation techniques, affinity reagents isolate only those Abs that interact with the immobilized ligand. Alternatively, affinity reagents can be used for the selective removal of specific contaminants, or for the analytical capture and detection of molecules. Moreover, if the immobilized molecule is an enzyme, enzymatic modifications of ligates can occur.

There are many decisions to be made when developing an affinity technique. Generally, the first step is choosing the ligand; the choice of ligand guides all subsequent decisions. Next, a solid support, or matrix, must be chosen and an activation method appropriate for both the matrix and the ligand must be determined. The affinity reagent is synthesized by coupling the ligand to the activated matrix. Finally, once the affinity reagent has been made, the conditions for binding and eluting the ligate must be determined.

A matrix is any material to which a biospecific ligand may be attached. For an affinity technique to be successful, the correct ligand must be optimally attached to the best matrix. There are several factors to consider when deciding upon the best matrix for an application. The matrix should be available in convenient quantities from a reliable source. The matrix can be synthesized in the laboratory, in which case, it can be designed to meet the exact requirements of the application. However, most researchers use commercially available matrices since they are convenient to use, "expertly" prepared and well-characterized in terms of their ligand loading, swelling and other properties. In general, buying a matrix ends up being more economical than a custom synthesis.

Whether synthesized or purchased, the matrix should have easily derivatizable functional groups, such as amines, carboxylates or hydroxyls, and should have good mechanical and chemical stability. The greatest challenges to the integrity of a matrix occur during activation and coupling since the matrix is exposed to mixing and chemical reagents for extended periods. Thus, the integrity of the matrix must not be comprised by the activation and coupling methods employed. Furthermore, the matrix should not swell greatly when placed in the solvents used for the affinity application. Swelling can change the structure of the matrix and immobilized ligand may not be accessible. Moreover, since most affinity techniques use aqueous buffers, the matrix should be hydrophilic but not water soluble.

The loading capacity of the matrix is an important factor since the density and accessibility of the immobilized ligand can affect the successful use of the affinity reagent. If the interactions between the immobilized ligand and ligate are weak, a low

ligand density may not be successful. On the other hand, a high ligand density may increase nonspecific binding to the affinity reagent. Nonspecific binding is the adsorption of molecules that do not have targeted sites of interactions with the immobilized ligand; nonspecific binding can be caused by ion exchange effects, or the hydrophobicity of the matrix.

Most matrix materials are porous. Thus the size of the pores is important since ligand can be displayed on the interior surfaces of pores. The pores must be large enough to allow the ligate access. The ability of the ligate to access the interior of pores can be compromised by excess swelling of the matrix. Also, the pore size should have a uniform distribution in order to minimize effects from gel-diffusion.

The flow characteristics of the matrix material must be considered. Beaded materials with a uniform size will pack well in a column and the solvent will flow freely. Finally, the matrix should have minimal nonspecific interactions with other components present in the samples to be purified.

Matrix materials can be from natural or synthetic sources (127). Some examples of matrices based on natural sources are agarose, cellulose and controlled pore glass or silica. Agarose and cellulose are both polysaccharides and therefore, have many hydroxyl groups available for activation. Agarose has large pores which increase the area available for ligand immobilization. The structural stability of agarose can be increased by cross-linking the polymers. Different commercial preparations have different degrees of cross-linking and thus, different pore sizes. Cellulose, however, does not have the same porous structure as agarose. As a result, cellulose is generally used as a membrane.

Controlled pore glass is included as a natural source matrix due to the occurrence of silica in Nature. Controlled pore glass is much more rigid than either agarose or cellulose and must be chemically treated to introduce useful functional groups before activation.

Matrices based upon synthetic polymers generally have increased physical and chemical stability compared to their natural counterparts (127). Some examples of synthetic matrix materials include sephacryl, polystyrene, polyacrylamide derivatives and azalactone beads. Sephacryl is a mixture of a natural polymer, dextran, and a synthetic monomer. Sephacryl was originally designed for gel filtration, but can be applied to affinity techniques. The most common affinity application for polystyrene matrices is ELISAs, however, polystyrene beads can be used in column formats. Polyacrylamide matrices are chemically stable to a wide pH range and do not possess many nonspecific interactions; however, they have slow flow rates, poor mechanical stability and swell a great deal. Azalactone beads have good mechanical stability and a high loading capacity since the rate of coupling is greater than the rate of hydrolysis.

Once a matrix has been chosen, it must be activated. Activation is the process of chemically modifying a matrix so it will react to form a covalent bond with a ligand. The activation method used must be compatible with both the matrix and the ligand; it must attach the ligand efficiently and firmly such that neither the ligand nor the matrix is damaged and no nonspecific characteristics are introduced.

Several guidelines should be considered when choosing an activation method. In general, the matrix and activation method are adjusted to suit the ligand, rather than adjusting the ligand to suit the matrix. The result of ligand coupling should be a stable

leak-resistant bond. Ligand leakage is a prime concern and can be caused by chemical cleavage of the bond between the ligand and the matrix, chemical or mechanical cleavage of the matrix backbone or degradation of the ligand. Leakage can be avoided by choosing an appropriate activation method, pre-washing the matrix to remove fragments and working in an appropriate pH range. The activation method and resulting ligand-matrix bond should leave the matrix free of nonspecific effects; for instance, unreacted sites and matrix-ligand bonds should be left uncharged. Furthermore, the activation method should leave the porosity and other matrix properties unchanged. Finally, the activation method should allow rapid and efficient coupling of the ligand. If the rate of reaction with the ligand is faster than the rate of hydrolysis, high ligand loading will result, even if the ligand concentration in solution is low. This is important when the ligand is scarce or expensive.

Methods of activation include incorporating reactive groups into the matrix during its synthesis, adding reactive groups by co-polymerizing suitable monomers during matrix synthesis or modifying the matrix to form reactive groups (127). Matrices can be modified with cyanogen bromide, *N*-hydroxy succinimide esters, carbonyl diimidazole, carbodiimides, epoxides, and azalactone. Reductive amination is another popular activation method.

Cyanogen bromide activation is the most common activation method and works with any poly-hydroxy matrix to introduce cyanate esters and imidocarbonates (128). The advantages of this activation method are that it works with any hydroxyl containing matrix, couples both small and large ligands with primary amines, and it is simple, mild,

and reproducible. However, the resulting bond has a small but consistent leakage because the isourea bond formed is unstable. Moreover, at neutral pH, the isourea bond is positively charged which increases the nonspecific character of the affinity reagent. In contrast, the amide bond formed with matrices activated with *N*-hydroxysuccinimide esters, carbonyl diimidazole, carbodiimides, or azalactones is more stable and uncharged; thus, ligand leakage is minimal and there is no increase in the nonspecific character of the matrices (127). However, many commercial matrices use cyanogen bromide chemistry to attach these other activating groups to the matrices; thus, affinity reagents made from such matrices can also deteriorate over time.

Reductive amination can be used to couple aldehydes or ketones to amines (127). The resulting Schiff base is reduced to a stable alkylamine. Generally, the aldehyde or ketone groups are part of the matrix; however, they can be contributed by the ligand. An example of an aldehyde containing ligand is a reducing carbohydrate. Unfortunately, a potential pitfall of reductive amination for the immobilization of carbohydrates is that the integrity of the sugar residue undergoing the reaction is destroyed by the coupling method. If this residue is important for the interactions involved, the resulting affinity reagent may not be functional.

After coupling a ligand to a matrix, ligand presentation must be considered. In many instances ligands are not joined directly to the matrix; rather, a spacer arm is introduced. A spacer arm is a low molecular weight molecule used as an intermediary linker between a matrix and a ligand. Generally, spacer arms consist of linear hydrocarbon chains with ten or fewer carbon atoms and chemical functionalities at both

ends which allow for easy coupling to the matrix and to the ligand. The use of a spacer arm can be crucial for the success of the affinity reagent. Often the binding site of the ligate to be purified is buried and steric interactions with the matrix would interfere with any complex formation. Steric interference is a greater concern when small ligands are immobilized. A spacer arm would not significantly increase the distance between the binding site of an immobilized protein and the matrix. What spacer arms can provide for immobilized protein ligands is directed attachment; that is, spacer arms can control what sites of a protein ligand are coupled to the matrix, ensuring that the binding site remains accessible. Spacer arms change the hydrophilicity around the ligand and may introduce ion effects. As a result, the nonspecific character of the matrix may be increased.

The density of the immobilized ligand can affect the success of an affinity technique. A low ligand density may not be successful if the interactions between the immobilized ligand and the desired ligate are weak. Alternatively, a high ligand density may increase the nonspecific interactions of the affinity reagent.

Once the affinity reagent is prepared, the optimal conditions for promoting the binding and elution of the ligate must be determined. The formation of the affinity complex depends on the concentrations of the ligand and the ligate, the accessibility of the immobilized ligand, the affinity between the ligate and the ligand, and the efficiency of matrix regeneration. The buffers used can promote the specific interactions involved while reducing the nonspecific interactions. Some of the factors that affect binding are the presence of salts and metal ions, the molarity and pH. In general, physiologic sodium chloride (0.15M) can prevent nonspecific interactions due to

ion exchange effects and will help to stabilize proteins; higher salt concentrations can decrease severe ionic interactions. Metal ions must be included in buffers when complex formation is metal ion dependent.

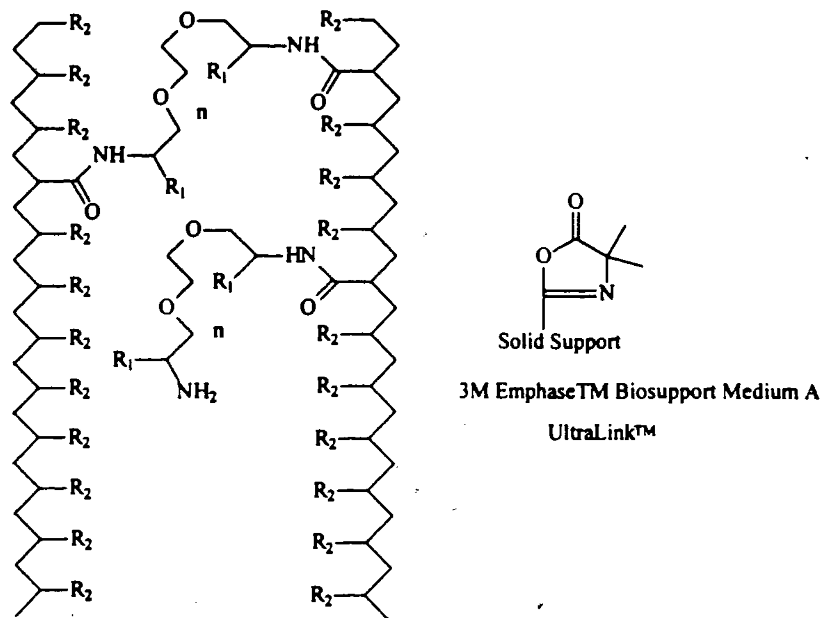
Once the ligate has been bound and the impurities washed away, the isolated ligate must be eluted from the affinity reagent. The ligate should be eluted in a minimum volume with its activity intact. The easiest elution method is to change the buffer conditions; increasing the salt concentration, varying the pH or adding metal ion chelators are common approaches (127). For most Ab:antigen interactions, the K_d is low; therefore, more drastic elution conditions may be required. Chaotropic agents such as guanidine or urea can be added. Since chaotropic agents change hydration shells, they work best for disrupting interactions that are mainly hydrophobic. The disadvantage of using chaotropic agents is that conformational changes occur which can lead to denaturation. Alternatively, the bound ligate can be displaced from the affinity reagent with a soluble counter ligand. However, if the K_d is low the concentration of the soluble ligand must be high.

Another factor to consider when developing an affinity technique is the length of time the ligate is exposed to the ligand. If the flow rate of a column is too fast, the ligate will not access the ligand bound on the interior of pores and the amount of ligate bound will be low. On the other hand, too slow a flow rate will be impractical and can lead to diffusion problems. In general, gravity flow is fine for affinity applications; however, it is common for Ab:antigen interactions to need more time. In these situations, the sample can be incubated on the column before elution.

5.2 Introduction to the Glycoconjugate Affinity Reagents

Dr. France-Isabelle Auzanneau used the Bra Tri (**4**) and the Penta (**7**) oligosaccharides corresponding to portions of the CWPS of GAS (see Figure 2.1) to prepare immunoaffinity reagents (36, 37). Two different matrices were used, the 3M Emphase™ Biosupport Medium AB 1 (UltraLink™, Pierce, Rockford, IL) and PEGA resins which are beaded polyethyleneglycol dimethylacrylamide co-polymers (129-131). Three different PEGA resins with different cross-linkers and differing degrees of cross-linking were prepared. The basic structure of these matrices are shown in Figure 5.1. Reductive amination, a common method of coupling carbohydrates to solid supports, was not used to couple the GAS oligosaccharides to the matrices since the first sugar residue would be destroyed, a significant change for these small ligands. Instead, cysteamine hydrochloride was reacted with the allyl glycosides of Penta (**7**) and Bra Tri (**4**) to produce a free amino group. This free amino group was used to couple Penta (**7**) directly to the azalactone-bearing UltraLink™ to give the affinity reagent **11** (36) (Figure 5.2). The free amino group was reacted with 3,4-diethoxy-3-cyclobutene-1,2-dione (diethyl squarate) (132) for coupling to the PEGA resins (37). The squarate derivatives were used to couple the oligosaccharides to the free amino groups present on the PEGA resins (Figure 5.3). These coupling methods take advantage of the carbohydrate intermediates already used in our laboratory for the synthesis of the GAS oligosaccharides (89, 90), preserve the entire hapten, and introduce a spacer arm.

The permeability of the PEGA resins had to be determined. Three different PEGA resins were prepared with different cross-linkers and differing degrees of cross-linking. It has been shown that the permeability of the PEGA resins depends upon the length of the cross-linker and the degree of molar cross-linking (37, 131). One PEGA resin, **12**, was prepared from a shorter cross-linker, polyethyleneglycol$_{1900}$ (PEG$_{1900}$), and had a 3% molar cross-linking (Figure 5.1). The other two PEGA resins, **13** and **14**, were prepared from a longer cross-linker, PEG$_{4000}$, and had 5% and 3% molar cross-linking, respectively (Figure 5.1). The squarate derivative of Penta (**7**) was coupled to resin **13**, to yield the affinity reagent **15**. The squarate derivative of Bra Tri (**4**) was coupled to resins **12**, **13** and **14** to yield the affinity reagents **16**, **17** and **18**, respectively (Figure 5.3) (37). The affinity reagents **15-18** were used to purify anti-oligosaccharide Abs from anti-Penta polyclonal sera in order to assess the usefulness of PEGA as a matrix for affinity reagents. The presentation and availability of the Penta (**7**) in the UltraLink™ affinity reagent **11** were also investigated.



PEGA resin 12 $R_1 = CH_3$ $R_2 = CONH_2$ n = 43-45
PEGA resin 13, 14 $R_1 = H$ $R_2 = CONH_2$ n = 77-100

Figure 5.1. The basic structures of the PEGA resins and UltraLink™.

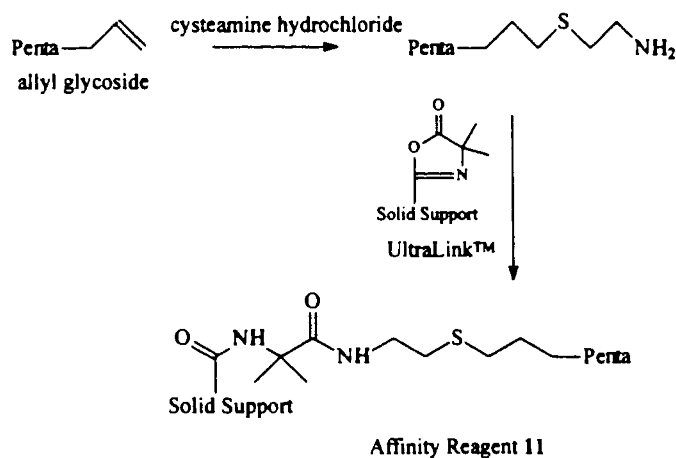
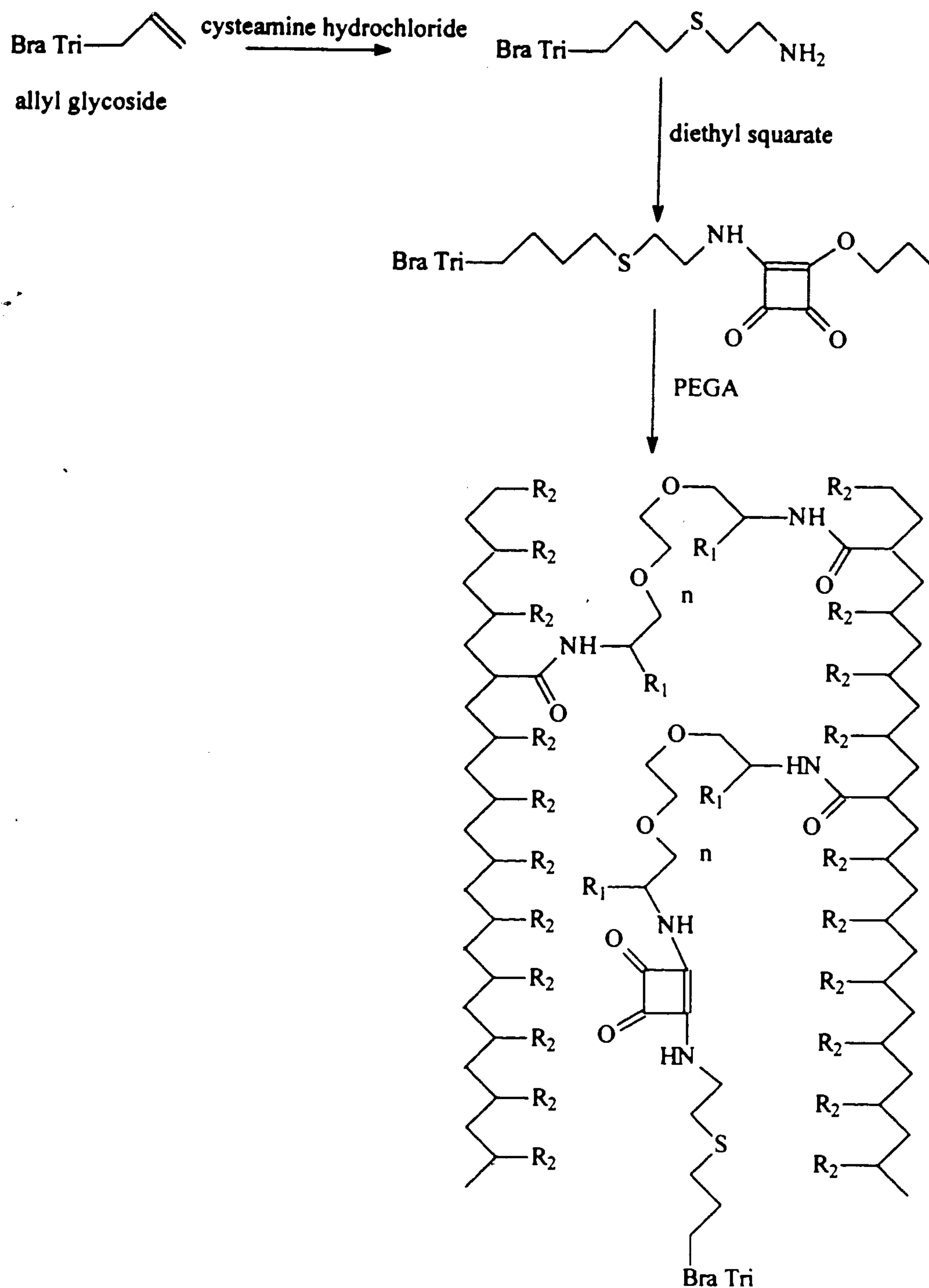


Figure 5.2. Synthesis of affinity reagent **11** (Penta (**7**) coupled to UltraLink™).



Affinity Reagent **16** $R_1 = CH_3$ $R_2 = CONH_2$ n = 43-45

Affinity Reagent **17, 18** $R_1 = H$ $R_2 = CONH_2$ n = 77-100

Figure 5.3. Synthesis of affinity reagents **16**, **17** and **18** (Bra Tri (**4**) coupled to PEGA resins **12**, **13**, **14**, respectively)

5.3 Results

5.3.1 Investigation of the availability and presentation of the Penta (7) chemically coupled to UltraLink™ (11).

The preparation of affinity reagent **11** is described elsewhere (36). The affinity reagent **11** was used to purify Abs that recognized the Penta (7) from the entire mixture of Abs in anti-Penta pcAb. The pcAb mixture contains Abs directed against the protein carrier used for the immunizations as well as Abs that recognize smaller portions of the Penta (7) hapten (**3**). In order for the affinity reagent to purify successfully the Penta (7) reactive Abs, the immobilized Penta (7) must be accessible and correctly presented.

Affinity reagent **11** contained 1.85 µmol of Penta (7) per mL of gel. A sample of caprylic acid-purified anti-Penta pcAb containing 420 µg of protein was added to a 650µL column containing affinity reagent **11**. Total protein concentration was approximated by determining the absorbance at 280 nm and assuming an extinction coefficient of 1.5. After washing away unbound protein, bound Abs were eluted with 1 M Glc*N*Ac in TBS. Approximately, 20 µg of Abs were recovered, as determined by A_{280} measurements; the yield of Abs eluted from the column was 5% of the total protein added.

Not all of the protein added to the column was expected to bind the immobilized Penta (7). Some of the Abs contained in the caprylic acid-purified sample may have been directed against portions of the Penta (7) that were not accessible on the immobilized ligand. Moreover, some serum proteins would also have been precipitated, and these proteins would not bind the Penta (7). Thus, the 5% yield may represent the fraction of

Penta (**7**)-reactive Abs present in the caprylic acid purified anti-Penta pcAb sample, rather than the binding capacity of the column. Therefore, the purification was repeated with a more concentrated sample in order to determine if the column's binding capacity was 20 μg, or if the amount of Penta (**7**)-reactive Abs in the entire mixture was 5%. Even when twice the original amount of protein was added to the column, the amount of Abs eluted was approximately 20 μg. Moreover, the binding remained was constant over several runs; thus, the binding of the column was not saturated over time since all bound protein was eluted. Thus, the binding capacity of affinity reagent **11** was 0.2 nmol per mL of gel.

The Abs purified by affinity reagent **11** were characterized by ELISA and compared to the Abs purified by caprylic acid precipitation alone or by protein A and BSA affinity columns. The abilities of the different Ab preparations to bind Lin Tri (**3**)-BSA, Bra Tri (**4**)-BSA, Penta (**7**)-BSA and dGAS were determined and the data are shown in Table 5.1. Purification of the caprylic acid-precipitated Abs with affinity reagent **11** reduced the background binding to BSA by 34%. As a result, the binding of the pcAbs, purified by the affinity reagent **11**, to the carbohydrate antigens was above background levels, while the binding of the caprylic acid precipitated pcAbs was not. However, even after purification on the affinity reagent **11**, the binding of the pcAbs to BSA was still significantly higher than for the pcAbs purified on a both a protein A and a BSA affinity column. Thus, while the Penta (**7**) immobilized on UltraLink^{TM} (affinity reagent **11**) is available for Ab binding, the best method for preparing pcAbs with low binding to BSA is to use both a protein A and a BSA affinity column.

Table 5.1. Binding of anti-Penta pcAb, purified by three different methods, to carbohydrate antigens.

Immobilized antigen	pcAb purified by		
	Caprylic acid precipitation and affinity reagent **11**	Caprylic acid precipitation	Protein A- and BSA-columns
Penta (7)-BSA	257[a]	237	223
Bra Tri (4)-BSA	270	242	242
Lin Tri (3)-BSA	270	250	256
dGAS	296	258	282
BSA	139	210	76

a) $(A_{405}-A_{490}) \times 1000$

5.3.2 Investigation of the availability of the Penta (7) chemically coupled to the PEGA matrix 13.

The preparation of the PEGA resins and the affinity reagents is described elsewhere (37). The affinity reagent **15** was used to purify Penta (7)-reactive Abs from the anti-Penta pcAb. Affinity reagent **15** contained 0.85 μmol of Penta (7) per mL of gel. A sample of caprylic acid purified anti-Penta pcAb containing 0.5 mg total protein was added. After washing away unbound protein with TBS, bound Abs were eluted with 1 M Glc*N*Ac in TBS. None of the fractions eluted with Glc*N*Ac exhibited an absorbance at 280 nm. In fact, the amount of protein collected during the initial wash was equal to the total amount of protein added to the column. Therefore, the immobilized Penta (7) was not available to interact with the Abs and no Ab was bound to this glycoconjugate column.

5.3.3 Investigation of the availability of Bra Tri (4) coupled to three different PEGA matrices.

PEGA resin **12** contained the shorter cross-linker PEG_{1900} and had a 3% molar cross-linking. The other resins, **13** and **14**, contained the longer cross-linker, PEG_{4000}, and had 5% and 3% molar cross-linking, respectively (37). (Resin **13** was used to make the Penta (7) containing affinity reagent **15**.) The resulting Bra Tri (**4**)-containing affinity reagents were labeled **16, 17** and **18**, respectively. Affinity reagent **16** contained 2.5 μmol of Bra Tri (**4**) per mL of gel, affinity reagent **17** contained approximately 0.8 μmol of Bra Tri (**4**) per mL of gel, and affinity reagent **18** contained 1.26 μmol of Bra Tri (**4**) per mL of gel.

The availability of the Bra Tri (**4**) chemically coupled to the different PEGA matrices was investigated by using these affinity reagents to purify Bra Tri (**4**)-reactive Abs from the anti-Penta pcAb. Bra Tri (**4**)-reactive Abs are present in the anti-Penta pcAb since the anti-Penta pcAb cross-reacts with the Bra Tri (**4**) hapten (**3**). Samples of caprylic acid purified anti-Penta pcAb containing 2.3 mg, 1.1 mg or 1.4 mg of total protein was added to columns containing affinity reagent **16, 17** or **18**, respectively. After washing away unbound protein with TBS, bound Abs were eluted with 1 M Glc*N*Ac in TBS.

For affinity reagent **17,** which used PEGA **13,** as did the Penta (7)-containing affinity reagent **15**, none of the Glc*N*Ac eluted fractions exhibited an absorbance at 280 nm. Moreover, the amount of protein collected during the initial TBS wash was equal to the total amount of protein added to the column. Therefore, as with affinity reagent **15,**

the Bra Tri (**4**) was not available to interact with the Abs and no Ab was bound. However, fractions eluted with Glc*N*Ac from affinity reagents **16** and **18** did contain protein, as determined by A_{280} measurements. The total amount of protein eluted from the column containing affinity reagent **16** was 70 μg while the column containing affinity reagent **18** eluted 90 μg of protein; 3% and 6%, respectively, of the total protein added to the columns. The columns could be reused without loss of binding since all bound protein was eluted. Thus, it appears that PEGA **13** is not permeable to Abs since no Abs bound affinity reagents **15** and **17**. The higher degree of molar cross-linking found in PEGA **13** may result in a pore opening that is too small to allow the passage of Abs. In contrast, the smaller degree of molar cross-linking in PEGAs **12** and **14** resulted in a resin permeable to Abs.

The Abs which were eluted with Glc*N*Ac from the Bra Tri (**4**)-containing affinity reagents **16** and **18** were compared to the Abs purified by caprylic acid precipitation alone or by a protein A and BSA affinity column. The binding of the different Ab preparations to Lin Tri (**3**)-BSA, Bra Tri (**4**)-BSA, Penta (**7**)-BSA and dGAS was determined by ELISA and the data are shown in Table 5.2. While purification with the affinity reagents **16** and **18** does decrease the background binding to BSA as compared to caprylic acid precipitation alone, the binding to BSA is still above the signal for dGAS and Lin Tri (**3**); however, signals for binding to Bra Tri (**4**) and Penta (**7**) could be detected. Abs purified with a protein A and a BSA affinity column have still lower background binding to BSA. Thus, while the Bra Tri (**4**) immobilized in affinity reagents **16** and **18** (which use PEGAs

12 and **14**, respectively) was available for Ab binding, the best method for preparing pcAbs with low binding to BSA is to use both a protein A and a BSA affinity column.

Table 5.2. Binding of anti-Penta pcAb, purified by four different methods, to carbohydrate antigens.

Immobilized antigen	pcAb purified by Caprylic acid precipitation and affinity reagent **16**	Caprylic acid precipitation and affinity reagent **18**	Caprylic acid precipitation	Protein A- and BSA-columns
Penta (**7**)-BSA	1354[a]	1364	1190	1185
BSA	696	724	1192	39
blotto	26	14	22	14
Lin Tri (**3**)-BSA	341	334	319	354
BSA	338	332	452	26
blotto	14	10	13	8
Bra Tri (**4**)-BSA	418	418	368	380
BSA	276	284	362	22
blotto	14	10	14	11
dGAS	122	117	116	202
BSA	658	676	1017	47
blotto	37	26	20	16

a) $(A_{405}-A_{490}) \times 1000$

5.4 Discussion

The observation that the Abs purified with the Bra Tri (**4**)-PEGA reagents **16** and **18** did not bind the Lin Tri (**3**) hapten nor dGAS was not unexpected. It has been demonstrated that while the anti-Penta pcAb binds the Bra Tri (**4**) and Lin Tri (**3**) haptens, the anti-Bra Tri pcAb does not bind the Lin Tri (**3**) hapten (3). Therefore, one would not expect Bra Tri (**4**)-reactive Abs to bind Lin Tri (**3**). Furthermore, the anti-Penta pcAb has

been shown to be better at binding dGAS than the anti-Bra Tri pcAb (Chapter 2); thus, it was not unexpected that Bra Tri (**4**)-reactive Abs purified from the anti-Penta pcAb were not able to cross-react with the native antigen present on dGAS (Table 5.2).

The three different PEGA resins contained cross-linkers with different lengths and/or different degrees of molar cross-linking. Resins **13** and **14** contained the longer cross-linker PEG_{4000}, while resin **12** contained the shorter cross-linker PEG_{1900}. However, resins **12** and **14** both had 3% molar cross-linking, while resin **13** had 5% molar cross-linking. Since the affinity reagents made from the resins with 3% molar cross-linking were able to bind Abs regardless of the length of the cross-linker, the permeability of PEGA resins to Abs depends more upon the degree of molar cross-linking than upon the length of the cross-linker.

The length of the cross-linker does, however, affect the permeability. The efficiency of the different affinity reagents can be compared by examining the ratio of eluted protein to total immobilized carbohydrate. This ratio is 12 μg protein per μmol of immobilized carbohydrate for the Penta (**7**)-UltraLink™ affinity reagent, **11**, 16 μg protein per μmol of immobilized carbohydrate for the Bra Tri (**4**)-PEGA affinity reagent **16** and 33 μg protein per μmol of immobilized carbohydrate for the other Bra Tri (**4**)-PEGA affinity reagent **18**. Thus, PEGA resins with a low degree of molar cross-linking and long cross-linkers have large pores that allow Abs access to immobilized ligands. Moreover, such PEGA resins provide better access to immobilized ligands than the commercially available UltraLink™. Finally, while it was possible to purify Abs using

glycoconjugate affinity reagents, the best method for purifying the pcAbs was the use of both a protein A and a BSA affinity column.

5.5 Methods

5.5.1 Caprylic Acid Precipitation of IgGs.

Four parts of sodium acetate (60 mM, pH 4) were added to one part of serum. The pH was adjusted to 4.5 with sodium hydroxide (5N) and caprylic acid was slowly added with vigorous stirring until 2.5% (volume/volume) was added. The mixture was stirred for 30 min at room temperature and then centrifuged (10 000 x g) for 30 min at less than 10°C. The supernatant was carefully collected and filtered through a 45 μm filter. One part of 10x phosphate buffered saline (PBS) was added to nine parts of filtered supernatant and the pH was adjusted to 7.4 with sodium hydroxide. The supernatant was cooled to 4°C and ammonium sulfate (saturated) was added until 45% saturation. The mixture was stirred for 30 min at 4°C and then centrifuged (7 000 x g) for 15 min at 4°C. The pellet was collected and resuspended in the original volume of PBS. The solution was dialyzed against PBS at 4°C overnight. The dialyzate was heated at 50°C for 20 min and centrifuged (5 000 x g) for 20 min to remove any precipitates.

5.5.2 Affinity Purification of Caprylic Acid Precipitated pcAb

An aliquot of caprylic acid-precipitated anti-Penta pcAb was added to a TBS (50 mM TrisHCL, 150 nM NaCl, pH 7.5) equilibrated column containing a glycoconjugate affinity reagent. The ratio of total protein added to immobilized carbohydrate was 3.4

nmol of protein per μmol of carbohydrate. After incubating the Abs on the column overnight at 4°C, the column was washed with five bed volumes of TBS. Fractions were collected and the A_{280} was measured. When no more protein washed off the column, bound Abs were eluted with five bed volumes of Glc*N*Ac (1M in TBS). Fractions were collected, the absorbance was measured at 280 nm vs. a 1M Glc*N*Ac blank. Fractions containing protein were combined and dialyzed overnight vs. TBS to remove the bound Glc*N*Ac. After concentrating the dialyzate, the A_{280} was measured.

5.5.3 ELISA.

Microwells were coated with Penta (**7**)-, Bra Tri (**4**)-, Lin Tri (**3**)-BSA (1 μg/well, diluted in TBS, 40 μL/well), dGAS (4 x 10^7 bacteria/well, diluted in TBS, 40 μL/well), BSA (2% in TBS) or blotto (5% milk powder in TBS) and incubated overnight at 4°C. The wells were washed three times with TBS and were blocked with blotto (200 μL) for 2 h at room temperature. After three washes with TBS, pcAb was added (100 nM in 0.2% BSA) and incubated for 4 h at room temperature. The wells were washed six times with TBS and bound Abs were detected with a secondary Ab conjugated to horseradish peroxidase (Pierce, Rockford, IL). Optical densities are reported as (A_{405}-A_{490}) x 1000.

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