Antibody combining sites and oligosaccharide determinants studied by competitive binding, sequencing and X-ray crystallography

David R. Bundle

Division of Biological Science, National Research Council of
Canada, Ottawa, Ontario, K1A 0R6, Canada.

Abstract - Synthetic oligosaccharides and polysaccharides of
known structure have been covalently attached to carrier
protein and enzymes for use in a strategy to generate and
select monoclonal antibodies with well defined carbohydrate
binding profiles. Direct competitive binding assays utilizing
antibody bound to a solid phase and saccharide-enzyme
conjugates provided convenient and reliable measurement of
specificity. Protocols were developed to prepare these
carbohydrate-enzyme conjugates. Antibodies specific for the
Brucella A and M polysaccharide/antigen were selected and
characterized by these techniques. The crystal structure of
an Fab fragment derived from one antibody has been solved at
2.7Å resolution. Preliminary crystal structure data in
conjunction with the amino acid sequence of light and F_d
polypeptide chains implicate tyrosine, arginine, asparagine,
glutamine and aspartic acid as the residues that contact
antigen. A second antibody-antigen system involving the
Shigella flexneri Y antigen has been studied by a similar
approach, although in this case two distinct antibodies have
been subjected to detailed binding studies with oligosaccharide
fragments of the polysaccharide repeating unit and specifically
modified derivatives. These data support the contention that
the principal polar interactions between an oligosaccharide
and its antibody involve a small group of hydroxyl residues and
that specifically deoxygenated oligosaccharide inhibitors can
have association constants significantly higher than the natural
oligosaccharide.

INTRODUCTION

Specific binding of carbohydrates by proteins has been the focus of increased
study during the last fifteen years. This activity reflects the
sophistication of instrumentation and experimental design in both
carbohydrate and protein chemistry. The most precise detail of protein-
carbohydrate complexes have come from X-ray crystallographic studies at high
resolution <2.0Å (ref. 1), although only a few protein structures have been
refined to this demanding level of resolution. Protein-saccharide systems
studied in greatest detail have been readily available enzymes (ref. 2-4),
lectins (ref. 5-8) and myeloma proteins (ref. 9 & 10). However,
increasingly, antibodies derived from somatic cell fusion (ref. 11) have been
studied (ref. 12-16) and genetically engineered antibodies and lectins have
recently been produced (ref. 17-19). The advantages offered by the latter
approaches reside both in the ability to select combining sites directed to
antigens of interest such as the human blood group antigens (ref. 12 & 13)
and, secondly, to specifically alter amino acid residues in the combining
site (ref. 18).

This paper reports the generation and selection of monoclonal antibodies by
the hybridoma technique (ref. 11), and the study of the combining sites by
X-ray cystallography and amino acid sequencing in combination with a
delineation of the structural reqirements and features of the combining sites
revealed by competitive inhibition studies utilizing synthetic
oligosaccharides. As a working hypothesis the following assumptions are made
(cf. ref. 14): - (i) the conformation of oligosaccharide in the bound state
closely resembles the multiple low energy conformers that are detected by
conformational analysis using high resolution ^{1}H and ^{13}C NMR spectroscopy

(ref. 20), (ii) these conformations are well represented by simple HSEA potential energy calculations (ref. 21 & 22) and molecular modelling of natural and modified inhibitors based upon such predictions are considered to hold-provided these are consistent with NMR data, (iii) the exo-anomeric effect, which is strengthened in aqueous environment (ref. 23) is such that to accommodate the carbohydrate, protein may undergo conformational changes (ref. 5 & 24).

Three antigen-antibody systems, each of which has yielded a crystalline antibody fragment (Fab) in the presence of oligosaccharide inhibitor, are the subject of study in this laboratory. These are:

    a)   Brucella A and M antigens,
    b)   Shigella flexneri Y polysaccharide,
    c)   Salmonella serogroup B polysaccharide.

These three antigens were chosen because all three contain interglycosidic linkages that involve only secondary hydroxyl groups, a condition that confines inter-residue flexibility to two torsional angles ϕ and ψ rather than the three torsional angles ψ,ψ and ω that are required to describe the degrees of freedom of 1,6 linkages. Investigations of the dextran and galactan binding monoclonal antibodies have dealt extensively with flexible antigens of this type (ref. 15 & 16). The expectation that the antigenic determinants of the Brucella, Shigella and Salmonella polysaccharides exhibit well defined solution conformations has been supported by NMR and molecular modelling studies (ref. 25-27). In addition, a wide variety of synthetic ligands representing the repeating units of each polysaccharide antigen have been prepared (ref. 28-30 and references cited therein). Studies of the Brucella and Shigella antigens and their complementary antibodies are the subject of this report.

The general features of the antibody combining site are well established (ref. 31 & 32) and the extent and specificity of oligosaccharide binding was revealed by early studies of linear dextrans (ref. 32), the human blood group antigens (ref. 33) and bacterial cell wall polysaccharides (ref. 34). Although current knowledge of the three dimensional structures of antibodies is based upon X-ray diffraction data, the number of structures solved by this technique remains small and the $\beta 1,6$ galactan binding myeloma J539 (ref. 35) is the only carbohydrate binding antibody for which a published structure is available. The binding site of this antibody has been subjected to extensive probing by synthetic oligosaccharides and specifically modified derivatives (ref. 36 & 37). Diffraction data has been collected for crystals of a ligand-antibody complex. The importance of the latter data cannot be overemphasized, since thermodynamic data is difficult to interpret without a well developed model of the protein combining site even when it involves as impressive an array of structurally modified ligands as those recently used to probe the blood group antigen binding sites of antibodies and lectins (ref. 6,7,13 & 14). Since a number of oligosaccharides have crystallized as a complex with the lectin IV of Griffonia simplicifolia, correlation of thermodynamic data of this type with a detailed crystal structure should soon be available (ref. 38).

The objective of the studies reported here has been to use a limited number of synthetic oligosaccharides to map the antibody binding site, and in conjunction with crystallographic studies of Fab fragments develop an unambiguous model that accounts for the specificity and energetics of the molecular recognition.

BINDING ASSAY AND SELECTION OF MONOCLONAL ANTIBODIES

The details of the hybridoma technique (ref. 11) for the production of monoclonal antibodies are well established and it is generally appreciated that speed, sensitivity and accuracy are the major considerations when selecting putative clones for further investigation. This must be accomplished approximately ten to twelve days after the fusion experiment, when it is not uncommon to be faced with the assay of culture supernatants from approximately 300 microculture wells. Lipopolysaccharide coated enzyme immunoassay (EIA) ·plates were used to identify polysaccharide specific antibodies and those wells exhibiting the highest colour response are recloned in the expectation that the strongest colour response not only reflects antibody concentration but also antibody affinity (ref. 39). This assay is often referred to as an indirect EIA or ELISA (Fig. 1a). Although well suited to rapid screening the assay response is dependent on both antibody concentration and avidity. The latter parameter is also dependent

Indirect Detection

A
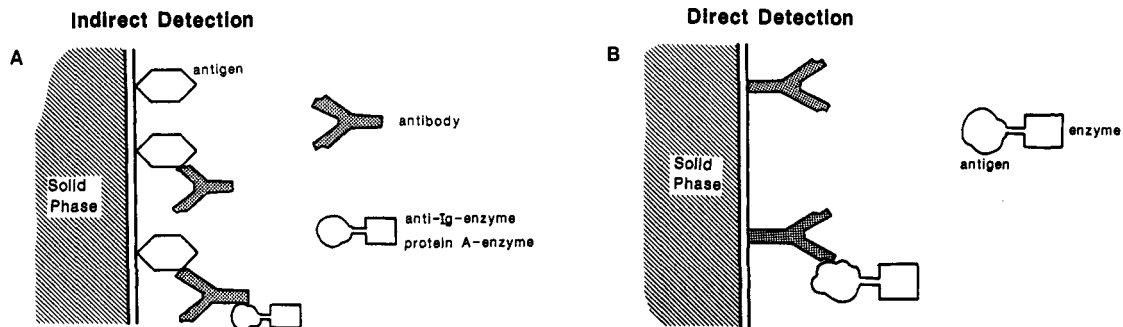
Direct Detection

B

Fig. 1. A) Indirect EIA with LPS antigen bound to the assay plate is used to detect specific antibody by using an enzyme linked to a goat anti-mouse immunoglobulin reagent. B) Direct detection uses antigen covalently attached to enzyme to provide the signals for bound antigen.

upon antibody class (IgM vs. IgG), and since clones of interest likely grow and secrete antibody at different rates, as well as belong to a different IgG subclass, a more discriminating assay is required for the final selection of hybrid-myeloma clones. For these reasons previous attempts to select antibodies on the basis of binding profiles with a range of glycoconjugates each containing a unique oligosaccharide determinant were only partially successful in selecting the highest affinity antibodies (ref. 40). These results agree with detailed investigations of the parameters affecting endpoint titrations determined by indirect EIA (ref. 41). Direct competitive EIA in which the antibody is bound to the assay plate and probed by labelled antigen (Fig. 1b) with or without added inhibitor provides a more reliable estimate of antibody affinity or, in the case of polyvalent antigens, avidity. Thus a <u>Brucella</u> specific antibody that bound the M and A polysaccharide antigens with nearly identical end-point titres was shown by direct competitive EIA to exhibit a four hundred fold higher avidity for the M relative to the <u>Brucella</u> A antigen (ref. 42).

CONJUGATES WITH SYNTHETIC OLIGOSACCHARIDES
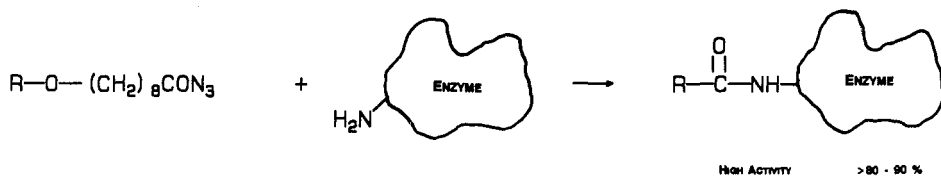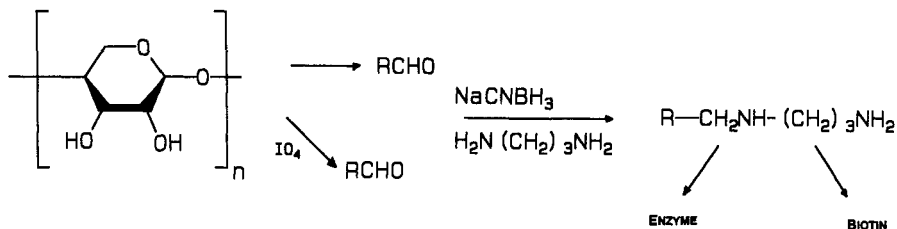
CONJUGATES WITH POLYSACCHARIDES

Fig. 2. Strategies to covalently link carbohydrates to enzymes. Synthetic oligosaccharides are coupled via a purpose built spacer. Polysaccharides are reacted at the reducing terminus or other aldehyde functions, introduced by mild periodate oxidation. Reductive amination directly with the enzyme (inefficient) or with 1,3-diaminopropane followed by bridging to an enzyme via a bifunctional spacer reagent yields active enzyme conjugates. Activated biotin was coupled directly to aminated polysaccharide.

In order to perform competitive assays of this type antigen labelled to a
high specific activity with a radioactive isotope of short half life are
often employed (ref. 43). Alternatively, enzyme labelled antigens may be
used (ref. 44). Although these types of conjugate have been found general use in
drug assay protocols, little if any effort has been made to prepare and use
carbohydrate-enzyme conjugates. Three types of conjugate have been prepared
to address this application (Fig. 2). Oligosaccharide glycosides of
8-methoxycarbonyloctanol were coupled to enzyme by mild and effective methods
(ref. 44) based upon the corresponding acyl azide (ref. 29). The mild
conditions employed provided an enzyme conjugate that retained at least 80%
of the original enzymic activity (Fig. 2). Attempts to conjugate bacterial
O-polysaccharides directly to enzymes by the reductive amination procedure
were of limited success (ref. 44). However, reductive amination in the
presence of a 1,3-diaminopropane spacer group gave a polysaccharide
derivative that could be coupled to enzyme under mild conditions via
established homo- and hetero-bifunctional linking reagents, such as
disuccinimidyl suberate (ref. 45) and sulfo-succinimidyl
4-(N-maleimidomethyl) cyclohexane-1-carboxylate (ref. 46) (Fig. 2). Enzyme
activity was retained but complications occurred if several polysaccharide
chains were bound to one enzyme with the result that multivalent binding to
solid phase antibody was no longer readily reversible by low concentrations
of active inhibitors (unpublished results). The solution to the problem of
magnified polyvalence was overcome by utilizing the avidin/biotin system
(ref. 47). Biotin N-hydroxysuccinimide ester was reacted with the aminated
form of the polysaccharide. The biotinylated polysaccharide was bound to the
solid phase antibody and in turn was detected by a horseradish
peroxidase-avidin conjugate (ref. 48). Since binding and inhibition are
established prior to cross-linking of bound antigen by the detection reagent,
the effects of polyvalence were circumvented (unpublished results).
Employing an assay of this type permitted a simple and rapid comparison of
relative inhibitory powers for a wide range of structurally related
oligosaccharides. In addition it was shown that at 50% inhibition the
concentration of a univalent inhibitor was inversely proportional to the
association constant, K_A (ref. 44).

Enzyme labelled antigens proved to be a reliable alternative to radiolabelled
polysaccharides or oligosaccharides (ref. 43) since they possessed a longer
shelf life. However, since the detection of antigen depends upon a secondary
reaction sensitive to factors associated with the realization of enzymic
activity, the enzyme based competitive EIA was found to give error bounds
twice as large as the comparable radioimmunoassay (ref. 39 & 43). This
corresponds to a standard deviation in the measurement of the relative
inhibitory power $\Delta(\Delta G) \pm 0.2$ Kcal/mole. The accuracy of this data is
considered to be well within the current capability to meaningfully account
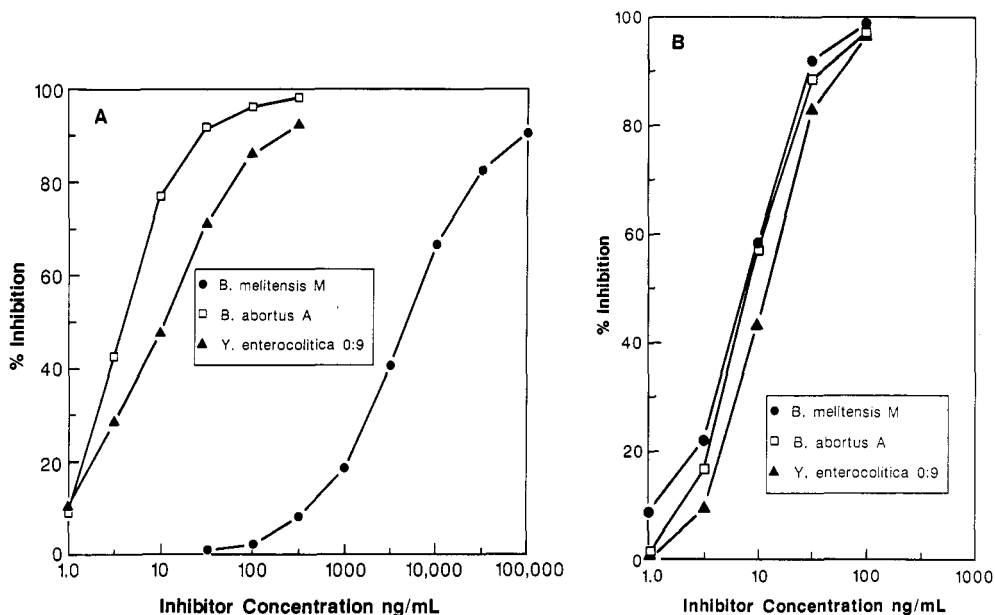for such small changes of binding energy.

Fig. 3. Competitive inhibition based upon solid phase antibody
binding biotin labelled A polysaccharide antigen and inhibited by the
three unlabelled polysaccharides A, M and Yersinia enterocolitica O:9
(similar structure to the A antigen). A) Antibody I. B) Antibody II.

BRUCELLA ANTIGENS AND ANTIBODIES

The <u>Brucella</u> A and M polysaccharide antigens are homopolymers of 4,6-dideoxy-4-formamido-D-mannose. Whilst the A antigen is an α1,2 linked polysaccharide (ref. 49), the M antigen is a polymer of linear pentasaccharide repeating units containing four α1,2 and one α1,3 linkages (ref. 50). Antibodies generated in BALB/c mice exhibit one of three binding profiles with the M and A antigens. The first two categories bind specifically to either the A or M antigen and the third binds both the A and M structures. Antibody I, an IgG$_{2b}$, binds the A antigen eleven hundred times more strongly than the M polysaccharide (Fig. 3a). The A and M antigens are bound with equal affinity by antibody II, an IgG$_3$ (Fig. 3b).
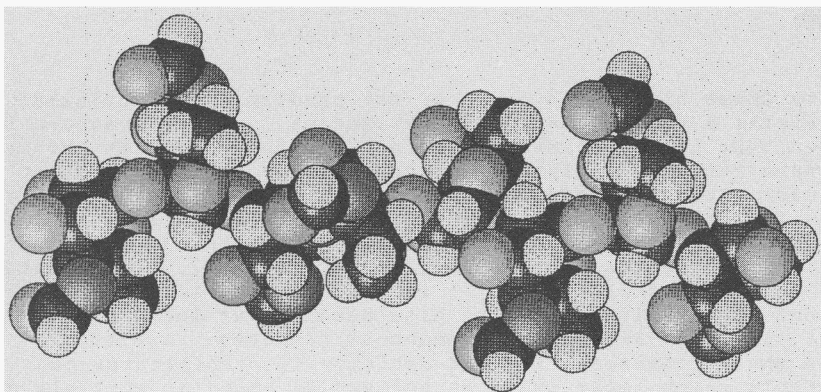

Fig. 4. The A antigen presented as an octasaccharide CPK model. The formamido moieties are observed at the periphery of the antigen's surface.

Removal of the formamido residues from either the A or M antigen followed by <u>N</u>-acetylation produced amino and <u>N</u>-acetylated polymers with functional affinities one thousand fold lower than the natural antigen. This implied that the formamido residue was an immunodominant feature (Fig. 4) although the possibility that either of its rotomeric forms may be populated in the bound state must be considered (ref. 51). Based upon the homology of the two antigens it was concluded that antibody II recognized an epitope common to the A and M polymers, and antibody I bound a distinctive A determinant. The most plausible explanation for these binding characteristics involved the size of the respective antibody combining sites. Antibody I required a pentasaccharide or possibly a hexasaccharide to express its full activity, whereas, antibody II possessed a combining site that was filled by a tetrasaccharide or smaller unit. These conclusions were supported by inhibition data obtained with synthetic oligosaccharides.

Oligosaccharide glycosides ranging from disaccharide up to pentasaccharide were synthesized starting from D-mannose (ref. 30 & 48). The approach utilized <u>1</u> as a key intermediate and adopted thioglycosides and methyl triflate activation for the formation of glycosidic linkages (ref. 30). Conversion of <u>1</u> to the thioglycoside <u>2</u> was accomplished via acetolysis and reaction of the 1,2-diacetate with ethanethiol in the presence of a Lewis acid. Reaction of <u>2</u> with bromine provided a mild method by which to prepare the glycosyl bromide <u>3</u> and this was required in order to glycosidate the thioglycoside alcohol <u>4</u>, derived from <u>2</u> by transesterification. The product of this reaction, the disaccharide thioglycoside <u>5</u> was a most effective derivative for block synthesis of the oligomeric series up to a pentasaccharide (ref. 30). Thus reaction of <u>5</u> with the glycosyl acceptor <u>6</u> yields a tetrasaccharide glycoside.
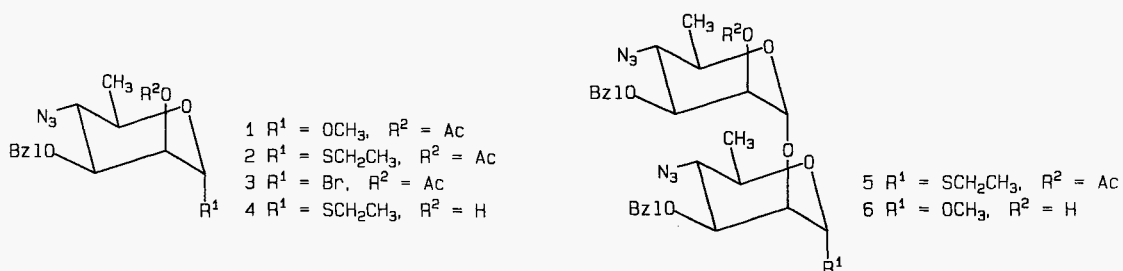

1 R^1 = OCH$_3$, R^2 = Ac
2 R^1 = SCH$_2$CH$_3$, R^2 = Ac
3 R^1 = Br, R^2 = Ac
4 R^1 = SCH$_2$CH$_3$, R^2 = H

5 R^1 = SCH$_2$CH$_3$, R^2 = Ac
6 R^1 = OCH$_3$, R^2 = H

| TABLE 1. Size of the Antibody II Combining Site | | |
Inhibitor Structure	Association Constant K_A (L/mole)	Relative free energy Kcal/ mole $\Delta(\Delta G)$
[Rha4NFo]$_2$-OMe	2.6 x 10^3	0.8
[Rha4NFo]$_3$-OMe	3.9 x 10^3	0.6
[Rha4NFo]$_4$-OMe	1.1 x 10^4	0.0
[Rha4NFo]$_5$-OMe	1.1 x 10^4	0.0

| TABLE 2. Size of the Antibody I Combining Site | | |
Inhibitor Structure	Association Constant K_A (L/mole)	Relative free energy (Kcal/ mole) $\Delta(\Delta G)$
[RhaNFo]$_2$-OMe	5.6 x 10^2	2.0
[RhaNFo]$_3$-OMe	8.2 x 10^2	1.7
[RHaNFo]$_4$-OMe	5.8 x 10^3	0.6
[RHaNFo]$_5$-OMe	1.6 x 10^4	0.0

Rh4NFo, 4,6-dideoxy-4-Formamido-
D-mannopyranose

Inhibition by these oligosaccharides of the binding of a biotinylated
derivative of the A polysaccharide to antibodies I and II absorbed to EIA
plates showed that the association constant K_A increased with increasing
inhibitor chain length. A plateau was reached for antibody II with the
tetrasaccharide glycoside and the pentasaccharide glycoside was only as
effective on a molar basis as the tetrasaccharide (Table 1). Antibody I
showed no plateau although the incremental binding for the addition of the
fifth pyranose residue was only 0.6 Kcal/mole (Table 2). These data support
the earlier inference that antibody I is able to discriminate the Brucella A
and M antigens because its combining site requires at least five contiguous
1,2-linked-4,6-dideoxy-4-formamido-D-mannose residues. Antibody I
precipitates the A polysaccharide and antibody II precipitates both the A and
M antigen, facts which imply that the antigen is bound at multiple sites
along the polymer chains. This type of binding necessarily requires the
polysaccharide to occupy a groove type combining site (ref. 32). This
conclusion appears to be supported by the crystal structure and modelling
studies.

X-RAY CRYSTALLOGRAPHY AND SEQUENCE STUDIES

Amino acid sequencing of the heavy (H) and light (L) chains derived from
antibody I has been completed (unpublished results). These data show that
the H chain is encoded by a germ line gene which is closely related to the
gene for the H chain of the myeloma protein MOPC 603 (ref. 52). The sequence
of the L chain resembles that found in hybridoma antibodies that bind the
p-phenylarsonate and the 2-phenyl oxazolone ligands (ref. 53). In fact, the
L chain shows highest homology with a human Bence-Jones protein REI. Since
the crystal structures of MOPC 603 and REI have been solved, we have
initiated modelling studies of the Fab, which makes use of these known
structures to infer the site of antibody I. This approach is proceeding in
parallel with the crystal structure study. Both sets of data suggest that
the polysaccharide is most likely bound at the surface of a shallow groove
formed between the H and L domains. Amino acids that are located at surface
positions in this region are tyrosine, arginine, asparagine, glutamine and
aspartic acid. It is tempting to speculate that contacts with these side
chains will provide binding features of the type reported for other
carbohydrate binding proteins (ref. 1), where planar dicarboxylic acids and
aromatic residues create binding surfaces in which hydrogen-bonds and van der
Waals interactions provide the specificity and energy of antigen binding.
The most striking feature of the groove between the H and L domains is the
presence of as many as eight tyrosine residues along its periphery. Crystals
formed in the presence of the pentasaccharide ligand have yet to attain a
size suitable for crystallographic analysis.

SHIGELLA Y POLYSACCHARIDE

This polysaccharide has been the subject of detailed investigations in our
laboratory both in synthesis and studies of solution conformation (ref. 25 &
28). The most relevant study with respect to this presentation is the
extension of the synthetic work to specifically modified and deoxygenated
oligosaccharides in an effort to identify that part of the antigen which is
most intimately bound in the antibody combining site of two monoclonal
antibodies prepared and selected by the type of approach described earlier
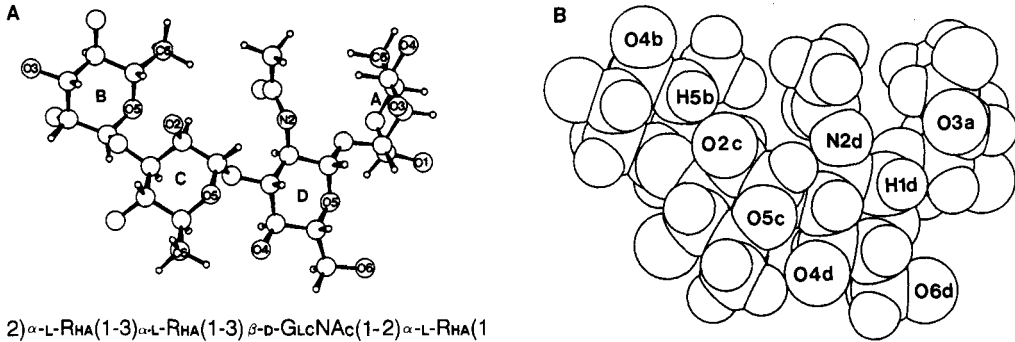and reported in published work (ref. 54).

2)α-L-RHA(1-3)α-L-RHA(1-3)β-D-GlcNAc(1-2)α-L-RHA(1

Fig. 5. A tetrasaccharide repeating unit of the <u>Shigella flexneri</u> Y
antigen with residue labelling BCDA (Rha-Rha-GlcNAc-Rha). A) Ball
and stick model, B) Space-filling model using van der Waal radii
reduced ~20% to facilitate presentation.

This objective was tackled in three stages. Firstly, since the antigen may
be bound at numerous surfaces along a polysaccharide chain composed of a
tetrasaccharide repeating unit (Fig. 5), the size and extent of the antigenic
determinant had to be identified. Secondly, the orientation of this unit
toward the protein surface was established and finally, attempts to identify
the critical polar contacts are near completion.

Competitive binding studies were performed by the EIA described earlier. The
Y-polysaccharide coupled to biotin or enzyme was allowed to compete with
inhibitor for solid phase antibody, either monoclonal GC-4 or SYA/J6, over an
18 h period. Assays were performed in either triplicate or duplicate and
relative inhibitory power was measured and compared at the point of 50%
inhibition. Under these conditions the association constant and hence free
energy of association could also be measured for univalent binding. The
frame shifted repeating unit BCDA (Fig. 5) (natural repeating unit ABCD) was
chosen as the reference point (Table 3). Changes in binding energy
can be seen for either the biological repeating unit ABCD or the
pentasaccharide ABCDA. Within the accuracy of the measurements these
differences cannot be considered significant. When the rhamnose unit B is
omitted the trisaccharide CDA shows 1.8 Kcal/mole lower binding energy, but
the difference of binding energy between the tetrasaccharide ABCD and the
trisaccharide BCD is only 0.6 Kcal/mole. The rhamnose trisaccharide ABC does
not bind indicating the essential role for the GlcNAc D residue. It was
concluded from these data that the tetrasaccharide BCDA represents the full
extent of the antigen surface in immediate contact with the antibody binding
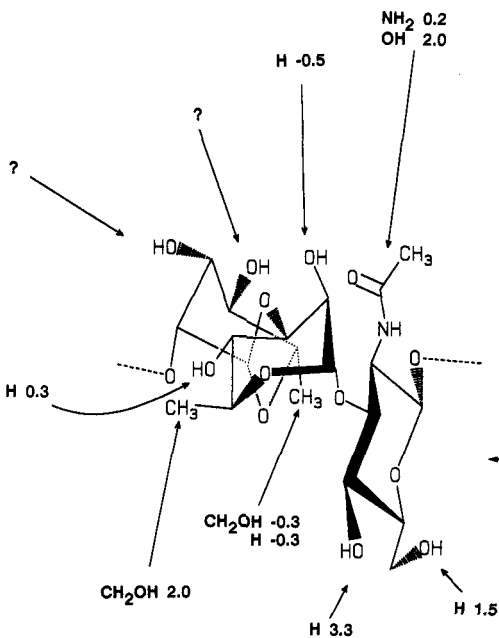site of the SYA/J-6 antibody.

TABLE 3. Location of the Antigen Binding
Surface for the <u>Shigella flexneri</u> Y
polysaccharide and SYA/J-6 antibody.

Inhibitor Structure					Relative free energy Δ(ΔG) Kcal/mole
Rha	Rha	Rha	GlcNAc	Rha	-0.1
Rha	Rha	Rha	GlcNAc		0.0
	Rha	Rha	GlcNAc	Rha	0.1
	Man	Rha	GlcNAc	Rha	0.6
	Rha	Man	GlcNAc	Rha	1.2

Fig. 6. The effects of specific single
site modifications of the trisaccharide
α-L-rha-(1→3)-α-L-rha-(1→3)-β-D-GlcNAc-OMe
on its binding to antibody GC-4. The
difference of free energy(Kcal/mole)
between the modified and unmodified
trisaccharide inhibitors is negative
for improved binding and positive for
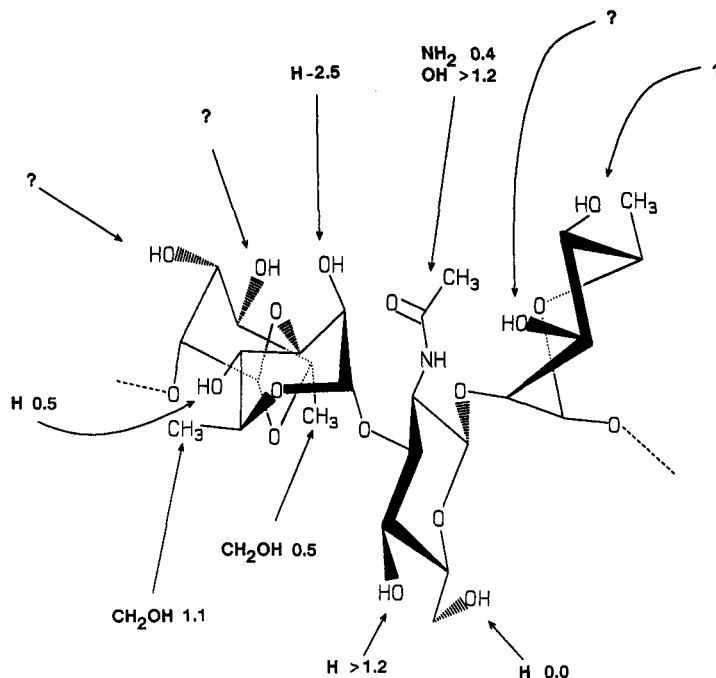inhibitors exhibiting lower binding
constants.

Fig. 7.   Single site modifications and their effect on binding of the
tetrasaccharide inhibitor α-L-(1→3)-α-L-Rha-(1→3)-
β-D-GlcNAc-(1→2)-α-L-Rha-OMe to SYA/J-6 antibody. The free
energy difference for each modification (Kcal/mole) is positive for
worse fit and negative for tighter binding.

The role of specific modification was investigated at the tetrasaccharide
level by incorporation of L-mannose for either rhamnose unit B or C.
This has the effect of introducing a sterically demanding and polar
hydroxylmethyl group in place of the non-polar methyl group. The free energy
changes for these alterations are shown for the antibodies GC-4 (Fig. 6) and
SYA/J6 (Fig. 7). Both antibodies showed only small changes in binding energy
for changes at C-6 of rhamnose B but the presence of a hydroxymethyl group at
C-5 of rhamnose C caused poorer binding by 0.5-1.0 Kcal/mole (Table 3). This
suggested that the tetrasaccharide surface in contact with protein included
C-6 of the rhamnose residue C and that the opposite edge of the
tetrasaccharide involving C-6 of rhamnose B and the acetamido function of the
GlcNAc residue D were more likely exposed to bulk solvent. In support of
this the amino derivative exhibited only a small loss of binding energy
relative to the acetamido form of residue D. The amide nitrogen does however
appear to be important since the replacement of the β-GlcNAc residue by Glc
results in a 1.2-2.0 Kcal/mole loss of binding energy.

The inferences concerning the antigen surface in contact with the protein
combining site were consistent with the data that aided the identification of
the principal polar contacts. These are most evident for the IgM antibody
GC-4 (Fig. 6). Deoxygenation at either C-4 or C-6 of the GlcNAc residue
causes substantial loss of binding most notably at C-4 > 3.3 Kcal/mole. By
comparison SYA/J6 showed O-4 of GlcNAc to be a crucial polar contact but not
O-6 (Fig. 7). Individual contributions from residue A which contributes in
the order of 0.5 Kcal/mole to binding have not been measured. Residue B
which is important to binding of SYA/J6 has yet to be investigated. The
contribution of residue C exhibits an interesting trend since for both
antibodies deoxygenation at C-4 causes at most a 0.5 Kcal/mole loss of
binding energy. However, the 2-deoxy rhamnose containing trisaccharide BCD
exhibits tighter binding than the natural tetrasaccharide sequence BCDA. The
magnitude of this change is 0.5 Kcal/mole for the GC-4 antibody but -2.5
Kcal/mole for the SYA/J-6 antibody (Fig. 6 & 7). The model proposed to
account for the data presented, envisages O-4 of the GlcNAc residue as a
crucial H-bonding residue in the combining site, which also involves O-6 in
the case of antibody GC-4. The acetamido residue is exposed to bulk solvent
and an extended non-polar surface is involved adjacent to O-4 of the D
residue and stretches from the α face of the GlcNAc residue to include the β
face of the α-L-Rha residue C. Replacement of the C-2C hydroxyl group by
a 2-deoxy function removes the only hydroxyl moiety that disrupts an

otherwise extensive non-polar surface (Fig. 5). This surface is conjectured to form important van der Waals contacts with the combining site and deoxygenation may enhance these contacts or remove the energetically expensive accommodation of an isolated hydroxyl group. The conformation depicted for the antigen and inhibitors (Fig. 5) are supported by ^{1}H and ^{13}C NMR data (ref. 25) and it is hoped that the proposed binding mode will be verified by X-ray diffraction studies of a crystalline Fab containing the pentasaccharide ligand ABCDA and the deoxygenated trisaccharide BCD. It is of interest that O-2 of rhamnose C is the site of O-acetylation in some Shigella antigens, a structural change with significant serological consequences (ref. 55).

SUMMARY AND FUTURE PROSPECTS

Brucella antibodies have been shown to possess combining sites specific for tetrasaccharide or pentasaccharide determinants. The X-ray structure of one such antibody implicates tyrosine and polar amino acid side chains as the most likely residues to contact the polysaccharide antigen. Two Shigella antibodies show combining sites of similar dimensions and probing by modified tetrasaccharide and trisaccharide inhibitors identified the O-4 and O-6 atoms of the GlcNAc residue as sites for potential H-bonding to protein. The removal of a hydroxyl residue from an otherwise exclusively non-polar surface of the inhibitor provides for binding energies higher than those of the unmodified ligand and in the case of one antibody this increase was ca. 2.5 Kcal/mole.

Manipulation of antibody binding sites to obtain improved fit of antigen has become a practical reality by employing the techniques of site specific mutagenesis and molecular modelling (ref. 18). It is also possible to transplant the hypervariable loops and obtain active antibodies to protein antigens (ref. 17). Using the amino acid sequence determined for the third antibody system - a Salmonella group B specific antibody (not discussed here) - we have initiated studies that are designed to produce a synthetic gene that codes for this antibody combining site (Narang and Bundle, work in progress). Using such approaches it should be possible to extend our understanding of carbohydrate-protein binding by studying modifications of the antibody combining site.

REFERENCES

1. F.A. Quiocho, Ann. Rev. Biochem., 55, 287-315 (1986).
2. E. Goldsmith and R.J. Fletterick, Pure and Appl. Chem., 55, 577-588 (1983).
3. I.P. Street, C.A. Armstrong and S.G. Withers, Biochemistry, 25, 6021-6027 (1986).
4. K. Bock, Pure Appl. Chem., 59, 1447-1456 (1987).
5. K.J. Neurohr, H.H. Mantsch, N.M. Young and D.R. Bundle, Biochemistry, 21, 498-503 (1982).
6. U. Spohr, O. Hindsgaul and R.U. Lemieux, Can. J. Chem., 63, 2644-2652 (1985).
7. O. Hindsgaul, D.P. Khare, M. Bach and R.U. Lemieux, Can. J. Chem., 63, 2653-2658 (1985).
8. K.A. Kronis and J.P. Carver, Biochemistry, 24, 834-840 (1985).
9. C.P.J. Glaudemans, Adv. Carbohydr. Chem. Biochem., 31, 313-346 (1975).
10. E.A. Kabat, J. Liao, M.H. Burzynska, T.C. Wong, H. Thorgersen and R.U. Lemieux, Mol. Immunol., 18, 873-881 (1981).
11. G. Köhler and C. Milstein, Nature, Lond., 256, 495-497 (1975).
12. D.R. Bundle,, M.A.J. Gidney, N. Kassam and A.F.R. Rahman, J. Immunol., 129, 678-682 (1982).
13. R.U. Lemieux, A.P. Venot, U. Spohr, P. Bird, G. Mandal, N. Morishima, O. Hindsgaul and D.R. Bundle, Can. J. Chem., 63, 2664-2668 (1985).
14. R.U. Lemieux, "The Hydrated Polar-Group 'Gate' Effect on the Specificity and Strength of the Binding of Oligosaccharides by Protein Receptor Sites", Proceedings of the VIIth International Symposium on Medicinal Chemistry, August 27-31, 1984, Uppsala, Sweden, Vol. 1, pp. 329-351.

15. C.P.J. Glaudemans, Mol. Immunol., 24, 371-377 (1987).
16. J. Sharon, E.A. Kabat and S.L. Morrison, Mol. Immunol., 19, 389-397
    (1982).
17. M. Verhoeyen, C. Milstein and G. Winter, Science, 239, 1534-1536 (1988).
18. S. Roberts, J.C. Cheetham and A.R. Rees, Nature, Lond., 328, 731-734
    (1987).
19. M.E. Stubbs, J.P. Carver and R.J. Dunn, J. Biol. Chem., 261, 6141-6144
    (1986) and Proc. of IXth Intl. Glycoconjugate Symposium, Lille, France,
    1987.
20. K. Bock, Pure and Appl. Chem., 55, 605-622 (1983).
21. R.U. Lemieux, K. Bock, L.T.J. Delbaere, S. Koto and V.S. Rao, Can. J.
    Chem., 58, 631-653 (1980).
22. H. Thogersen, R.U. Lemieux, K. Bock and B. Meyer, Can. J. Chem. 60, 44-57
    (1982).
23. J.-P. Praly and R.U. Lemieux, Can. J. Chem., 65, 213-223 (1987).
24. R. Zidovetzki, Y. Blatt, G. Schepers and I. Pecht, Mol. Immunol., 25,
    379-383 (1988).
25. K. Bock, S. Josephson and D.R. Bundle, J.C.S. Perkin Trans. II, 59-70
    (1982).
26. K. Bock, M. Meldal, D.R. Bundle, T. Iversen, B.M. Pinto, P.J. Garegg,
    I. Kvanström, T. Norberg, A.A. Lindberg and S.B. Svenson, Carbohydr.
    Res., 130, 35-53 (1984).
27. D.R. Bundle, J.W. Cherwonogrodzky and M.B. Perry, Biochemistry, 26,
    8717-8726 (1987).
28. B.M. Pinto, D.G. Morrisette and D.R. Bundle, J.C.S. Perkin Trans I, 9-14
    (1987).
29. B.M. Pinto, and D.R. Bundle, Carbohydr. Res., 124, 313-318 (1983).
30. T. Peters and D..R. Bundle, J.C.S. Chem. Comm., 1648-1650 (1987).
31. P.M. Alzari, M.-B. Lascombe and R.J. Poljak, Ann. Rev. Immunol., 6,
    555-580 (1988).
32. E. A. Kabat, Adv. Protein Chem., 32, 1-75 (1978).
33. E.A. Kabat, 'Structural Concepts in Immunology and Immunochemistry' Holt,
    New York, 1976, 2nd. Edn.
34. O. Luderitz, A.M. Staub and O. Westphal, Bacteriol. Rev., 30, 192-255
    (1966).
35. S.W. Suh, T.N. Bhat, M.A. Navia, G.H. Cohen, D.N. Rao, S. Rudikoff and
    D.R. Davies, Proteins, 1, 74-80 (1986).
36. C.P.J. Glaudemans, P. Kovac and K. Rasmussen, Biochemistry, 23, 6732-6736
    (1984).
37. C.P.J. Glaudemans and P. Kovac, Mol. Immunol., 22, 651-653 (1985).
38. M. Vandonselaar, L.T.J. Delbaere, U. Spohr and R.U. Lemieux, J. Biol.
    Chem., 262, 10848-10849 (1987).
39. F. Peterfy, P. Kuusela and O. Makela, J. Immunol., 130, 1809-1813
    (1983).
40. N.I.A. Carlin, D.R. Bundle and A.A. Lindberg, J. Immunol., 138, 4419-4427
    (1987).
41. A.M. Lew, J. Immunol. Methods, 72, 171-176 (1984).
42. D.R. Bundle, J.W. Cherwonogrodzky, M.A.J. Gidney, J. Meikle, M.B. Perry
    and T. Peters, Infect. Immun., submitted.
43. W.W. Young, Jr., H.S. Johnson, Y. Tamura, K.-A. Karlsson, G. Larson,
    J.M.R. Parker, D.P. Khare, U. Spohr, D.A. Baker, O. Hindsgaul and R.U.
    Lemieux, J. Biol. Chem., 258, 4890-4894 (1983).
44. E. Vorberg and D.R. Bundle, J. Immunol. Methods, submitted; E. Vorberg,
    P.J. Meikle and D.R. Bundle, Proc. of IXth Intl. Glycoconjugate
    Symposium, Lille, France, 1987.
45. P.F. Pilch and M.P. Czech, J. Biol. Chem., 254, 3375-3381 (1979).
46. M.J. O'Sullivan, E. Grammi, D. Morris, G. Chieregatti, M. Simmons, A.D.
    Simmons, J.W. Bridges and V. Marks, FEBS Lett., 95 311-313 (1978).
47. M. Wilchek and E.A. Bayer, Immunology Today, 5, 39-43 (1984).
48. R. Rappuoli, P. Leoncini, P. Tarli and P. Neri, Anal. Biochem., 118,
    168-172 (1981).
49. M. Caroff, D.R. Bundle, M.B. Perry, J.W. Cherwonogrodzky and J.R. Duncan,
    Infect. Immun., 46, 384-388 (1984).
50. D.R. Bundle, M. Gerken and T. Peters, Carbohydr. Res., 174, 239-251
    (1988).
51. L. Keene, P. Unger and T. Wehler, J.C.S. Perkin Trans. I, 1183-1186
    (1988).
52. S. Crews, J. Griffin, H. Huang, K. Calame and L. Hood, Cell, 25, 59-66
    (1981).
53. C. Berek, G.M. Griffith and C. Milstein, Nature, Lond., 316, 412-418
    (1985).
54. N.I.A. Carlin, M.A.J. Gidney, A.A. Lindberg and D.R. Bundle, J. Immunol.,
    137, 2361-2366 (1986).
55. L. Kenne, B. Lindberg, K. Petersson, E. Katzenellenbogen and E.
    Romanowska. Eur. J. Biochem. 91, 279-284 (1978).