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Anti-idiotype antibodies and phage displayed peptides as antigenic mimics of a *Mycoplasma capricolum* subsp. *capripneumoniae* epitope.

by

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Dedication

In loving memory of

Pookie

and the endless joy and love she gave me.

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Summary

Contagious caprine pleuropneumonia (CCPP) is a highly contagious disease that affects goats in Africa and Asia resulting in great economic losses. The aetiological agent is *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp). It belongs to a group of organisms that are all associated with economically important diseases of ruminants and all exhibit similar genetic and antigenic characteristics. The diagnosis of CCPP has often been considered difficult due to confusion with other mycoplasmas of ruminants and the limited specificity of most diagnostic assays. It is therefore important to improve diagnosis and thereby the control of CCPP. Two approaches, namely phage display and anti-idiotype antibodies can be used to identify antigenic mimics with the potential to be used in developing new vaccines, diagnostic methods or therapeutics. Both were used in an attempt to generate surrogate antigens for Mccp.

A monoclonal antibody (Mab) or its $F(ab')_2$ fragments directed against Mccp membrane protein epitopes was injected into hens to induce the production of anti-idiotype antibodies. The antibodies produced were functionally able to mimic the epitope recognised by the Mab since they inhibited binding of the Mab to mycoplasmal lysate. Mice were also immunised with the Mab and or $F(ab')_2$ fragments of the antibody. The resulting antisera were tested in ELISA, but no significant response was detected.

The selection of peptides from a random epitope library displayed on the surface of filamentous phages was used to characterise the epitope recognised by the Mab. Two different, but related peptides were identified that reacted with the antibody in enzyme-linked immunosorbent assays (ELISA). Binding to the Mab was further characterised by surface plasmon resonance. Sequence analysis revealed that the two peptides each had a cysteine residue in addition to the one fixed in amino acid position 2 as well as identical or similar amino acid residues in positions 5 (P), 8 (I/L) and 13 (L). One of the peptides had 74% similarity with an amino acid sequence of the PG1 strain of *Mycoplasma mycoides* subsp. *mycoides* SC.

The peptides as well as the anti-idiotype antibodies were not detectable using Mccp-specific goat antiserum suggesting that the serum did not contain paratopes that could accommodate the surrogate epitopes. In spite of this, the antiserum efficiently inhibited the binding of the Mab to immobilised mycoplasmal lysate in a standardised test for Mccp antibodies. This assay therefore appears to depend on a structural, rather than a functional blocking of the epitope which the Mab recognises. The findings in this study have elucidated some of the characteristics of the Mab and raised the possibility that the epitope recognised by the Mab is not immunodominant. Peptides identified by phage display and chicken/murine anti-idiotype antibodies, nevertheless, have the potential of being used as antigens in immunoassays aimed at CCPP diagnosis. In light of the results generated it would therefore be necessary to investigate other Mabs or polyclonal antiserum in order to yield antibodies or peptides of the desired specificity.

Abbreviations

µg	-	microgram
µl	-	microlitre
µm	-	micrometre
^{35}S	-	sulphur-35 isotope
°C	-	degrees Celsius
A	-	absorbance
Ab	-	antibody
Ab2	-	anti-idiotype antibody
Ag	-	antigen
bp	-	base pair
BSA	-	bovine serum albumin
CCPP	-	contagious caprine pleuropneumonia
cDNA	-	complementary deoxyribonucleic acid
CDR	-	complementarity determining region
CFT	-	complement fixation test
CFU	-	colony forming unit
cm	-	centimetre
Da	-	dalton
dATP	-	deoxy-adenosine triphosphate
ddA	-	2',3'-dideoxy-adenine
ddC	-	2',3'-dideoxy-cytosine
ddG	-	2',3'-dideoxy-guanine
ddT	-	2',3'-dideoxy-thymine
DF	-	dilution factor
DNA	-	deoxyribonucleic acid
EDC	-	*N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide
ELISA	-	enzyme-linked immunosorbent assay
$F(ab')_2$	-	bivalent antigen binding fragment of an antibody
Fab	-	monovalent antigen binding fragment of an antibody
Fc	-	non-antigen binding fragment of an antibody
g	-	force of gravity

g	-	gram
GIT	-	growth inhibition test
HRP	-	horseradish peroxidase
IFA	-	indirect fluorescent antibody
IgG	-	class G immunoglobulin
IgY	-	class Y immunoglobulin
IHA	-	indirect haemagglutination
i.p.	-	intraperitoneal
K_A	-	association equilibrium constant
kbp	-	kilobasepair
K_D	-	dissociation equilibrium constant
kDa	-	kilodalton
LB	-	Luria-Bertani
LC	-	large colony
M	-	molar
Mab	-	monoclonal antibody
Mccp	-	*Mycoplasma capricolum* subsp. *capripneumoniae*
mCi	-	milliCurie
mg	-	milligram
MHC	-	major histocompatibility complex
min	-	minute
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
mol	-	mole
M_r	-	molar mass ratio
mRNA	-	messenger ribonucleic acid
MW	-	molecular weight
N	-	normal
NAP	-	sodium chloride-ammonium phosphate
ng	-	nanogram
NHS	-	*N*-hydroxysuccinimide
nm	-	nanometre
nov.	-	new (refers to species)

OD	-	optical density
OPD	-	o-phenylenediamine dihydrochloride
OVI	-	Onderstepoort Veterinary Institute
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PEG	-	polyethylene glycol
pH	-	the negative of the base-10 logarithm of the hydronium ion concentration
pI	-	isoelectric point
pIII	-	phage coat protein III
pmol	-	picomole
PSB	-	protein solvent buffer
pVIII	-	phage coat protein VIII
RBC	-	red blood cell
RF	-	replicative form
rRNA	-	ribosomal ribonucleic acid
rpm	-	revolutions per minute
rRNA	-	ribosomal ribonucleic acid
RU	-	resonance unit
SC	-	small colony
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPR	-	surface plasmon resonance
ssDNA	-	single stranded deoxyribonucleic acid
subsp.	-	subspecies
TAE	-	Tris-sodium acetate-ethylenediamine tetraacetic acid
TBE	-	Tris-boric acid-ethylenediamine tetraacetic acid
TBS	-	Tris buffered saline
TE	-	Tris-ethylenediamine tetraacetic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
TGS	-	Tris-glycine-sodium dodecyl sulphate
Tris	-	tris(hydroxymethyl)-aminomethane
TU	-	transducing unit
V	-	voltage

W - watt

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Figure 3.4.12

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Chapter 1

Review of literature and introduction

Science knows only one commandment : contribute to science.

Bertolt Brecht, 1898-1956

1.1 Contagious caprine pleuropneumonia

1.1.1 Characteristics of the disease

The earliest documentation of contagious caprine pleuropneumonia (CCPP) (Hutcheon, 1881) describes a specific contagious disease in an epidemic form in a goat population where it caused the death of 700 animals in two weeks in Port Elizabeth, South Africa. The disease seems to have been introduced by a shipment of goats from Angora, Turkey. Efforts to isolate the aetiological agent commenced in the 1900s. The first significant transmission studies with a caprine mycoplasma which caused pleuropneumonia were described by Beaton in 1931 (for a review, see MacMartin, 1980). He found that the disease was spread naturally by contact with susceptible goats, but was unsuccessful in reproducing the disease experimentally. It appeared that his isolate (obtained from Nigeria) was in fact not the causative agent of CCPP. For many years a highly significant isolate was the strain named PG3 (for a review, see MacMartin, 1980), which was used as the reference strain for mycoplasmas isolated from goat pneumonias. There was, however, no description of the case or outbreak from which the material was taken and only two goats were used to determine the pathogenicity of the isolate. It did not produce any of the characteristics of classical CCPP. MacOwan and Minette (1976) were the first to successfully transmit CCPP to healthy goats which were inoculated with mycoplasma strain F38, isolated from the lung lesions of goats with acute CCPP in Kenya. The clinical signs and the ease with which the disease spread to healthy goats suggested that Hutcheon's classical disease syndrome had finally been reproduced and the new organism isolated.

CCPP appears to be confined entirely to goats. It is widespread on the African and Asian continents, but has also been reported in Europe (Thiaucourt and Bolske, 1996). The disease is spread by close contact with an infected animal where transmission is direct via the aerogenic route. Some animals may become latent chronic carriers and thus play a role in the spread of the disease, but the exact location of *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) in latent carriers is unknown.

Pathological changes in goats suffering from CCPP are confined to the chest cavity and consist of pleuropneumonia, i.e. unilateral hepatisation, an accumulation of straw-coloured pleural fluid and acute pleuritis adjacent to the affected lung. The lungs are enlarged, firm and oedematous, varying in colour from red to grey, forming a mosaic. Sections appear 'granular' with various colours, but without any thickening of the interlobular septa. Evolving lesions are characterised by round foci of hepatisation with a grey pinpoint centre of necrotisation and dark red hyperaemic margins, which contrast markedly with the pink unaffected lung (Thiaucourt *et al.*, 1996).

1.1.2 Taxonomy

The early confusion notwithstanding, MacOwan and Minette's (1976) Mycoplasma strain F38 was established as the true causative agent of classical CCPP displacing other mycoplasmal candidates previously associated with this disease (for a review, see McMartin *et al.*, 1980). Taxonomic studies established that F38 belonged to the class *Mollicutes*, genus *Mycoplasma,* but was apparently distinct from previously described members of the genus, except for some serological cross-reactivity with a few species (Ernø *et al.*, 1979; 1983). The most important cross-reactions were with some of the five mycoplasmas which, together with the F38 group, had began to emerge as a potential taxonomic group, the so-called *Mycoplasma mycoides* cluster. This cluster includes the two biotypes of *M. mycoides* subsp. *mycoides* (the small-colony and large-colony forms), *M. mycoides* subsp. *capri*, *Mycoplasma capricolum*, bovine serogroup 7 and the F38 group (Leach, 1973). Priority was given to resolving the relationship between *M. capricolum* and the F38 group due to their close serological similarity and the high DNA-DNA relatedness value (80%) reported for the type strain of *M. capricolum* and strain F38 by Christiansen and Ernø (1982). After examining five isolates of each taxon in DNA-DNA hybridisation experiments and considering the cultural, biochemical and serological properties of the two groups, a subspecies relationship between the two taxa have been proposed (Bonnet *et al.*, 1993; International Committee On Systematic Bacteriology, 1991a; 1991b). In light of this proposal, F38 and related strains were designated as *M. capricolum* subsp. *capripneumoniae* subsp. nov. and *M. capricolum* strains as *M. capricolum* subsp. *capricolum* subsp. nov.

1.1.3 Structure and genome

Although typical prokaryotes usually have a peptidoglycan containing cell wall, Mccp, like the other members of the class *Mollicutes,* is bounded only by a single trilaminar cell membrane. Electron microscopy reveals pleomorphic coccoidal forms 300 to 850nm in diametre to short or long, branched or unbranched filaments 200 to 300nm in width to 1500nm in length. It produces colonies having a classical 'fried egg' appearance only after five days of aerobic growth with a maximum colony size of 0.5mm after seven days (Ernø *et al.*, 1983). Its base composition (G+C) of 24.4% (Christiansen and Ernø, 1982), sensitivity to digitonin, the requirement of cholesterol for growth, lack of spiral morphology and inability to hydrolyse urea further classify Mccp within the genus *Mycoplasma.* It appears to possess a capsule-like structure composed of equal quantities of glucose, galactose, mannose, fructose, galactosamine and glucosamine (Rurangirwa *et al.*, 1987c). The polysaccharide produced by *Mycoplasma mycoides* subsp. *mycoides* (small colony form), the causative agent of CBPP and a species closely related to Mccp, diffuses readily in the tissues of infected cattle and large amounts are found circulating in blood, lung, pleural fluid and urine (for a review, see Nicholas and Bashiruddin, 1995).

Despite its importance, more is known about other animal and human mycoplasmas from which characteristics of Mccp can be extrapolated. The size of the genome in the genus *Mycoplasma* varies from 600 to 2200kbp (International Committee On Systematic Bacteriology, 1995), which is approximately one-fifth of that of *Escherichia coli.* This is thus the smallest genome known for a self-replicating prokaryote. Unlike most bacteria, mycoplasmas use the UGA codon to code for tryptophan rather than as a stop codon. It is therefore difficult to express cloned mycoplasma genes in most normal bacterial hosts (Yamao *et al.*, 1985). It has also been reported that some mycoplasmal DNA polymerases lack the 3'-5' exonuclease activity that provides normal bacteria with a proof-reading function. As a consequence, mycoplasmas are more liable than other bacteria to synthesise new DNA strands incorrectly (Maurel *et al.*, 1989) and this leads to a high genomic mutation rate (Lee *et al.*, 1987). These mechanisms are evident from the wide variation in the genome size of strains of some members of the *Mycoplasma mycoides* cluster.

1.1.4 Pathogenicity and pathogenesis

The vast majority of *Mollicutes* are cell-surface parasites, which rarely invade or enter the circulation and are generally confined to the mucosal or epithelial surfaces lining the oral cavity, synovial tissue or urinogenital tract (Tully, 1992). A striking feature of Mccp is its strict host and tissue specificity, as lesions are exclusively produced in goat lungs. Galactan has long been associated with the pathogenicity of *M. mycoides* subsp. *mycoides* but its mode of action remains unknown. The results of Lloyd *et al.*, (1971) suggested that the amount of galactan produced by a strain of *M. mycoides* subsp. *mycoides* was directly proportional to its virulence. Mccp also produces a polysaccharide, but its role in pathogenicity is unknown. It does however react with antibodies in sera from goats suffering from CCPP and consists of glucose, galactose, mannose, fructose, glucosamine and galactosamine in approximately equal quantities (Rurangirwa *et al.*, 1987c).

M. pneumoniae and *M. genitalium* are human pathogens with specialised terminal structures that have been shown to carry adhesins, proteins that may mediate attachment to eucaryotic cells and tissues (Razin and Jacobs, 1992), but no such component has been described for Mccp. These mycoplasma species can also alter the structure of their membrane surface in order to avoid destruction by the host's immune response (Wise, 1992). Very little information exists about the pathogenic mechanisms of Mccp making further studies vital in order to understand the mechanisms involved.

1.1.5 Diagnosis

Isolation:

Detection and/or identification of Mccp is hampered by its similarity to the other members of the *Mycoplasma mycoides* cluster. For instance, it shows strong serological cross-reactivity with *M. capricolum* subsp. *capricolum* and *Mycoplasma* species bovine serogroup 7 (Ernø *et al.*, 1983; Kanyi Kibe *et al.*, 1985; Wamwayi *et al.*, 1989; Guerin *et al.*, 1993; Thiaucourt *et al.*, 1994). Diagnosis of the disease is assisted by microscopic examination of lung material, but a definitive diagnosis requires culture of the causative organism from lung tissue samples taken at post-

mortem. The isolation of Mccp is usually a long and difficult process. Growth is very poor *in vitro* and after four to five days of incubation the resulting colonies can only be seen with a binocular microscope as they may only be 0.1mm in diameter. Purification of the isolates is crucial and is performed by repeated transfer of single colonies representing each morphological type seen to either fresh agar or broth media. Colony morphology, however, varies with the medium used, the mycoplasma species, its passage level and the age of the culture. In early passage many mycoplasma species including Mccp produce bizarre colony morphology, often small, centreless and of irregular shape. Only with passage do these isolates demonstrate the conventional 'fried-egg' colony morphology characteristic of mycoplasmas and which do not adhere to the agar surface (Razin, 1983). Filtration of broth cultures through 0.45µm filters before subculture aids in the purification by excluding cell aggregates. The addition of horse (Thiaucourt *et al.*, 1996) or foetal calf serum (Jones and Wood, 1988) aids in the growth of these organisms. Another major difficulty in Mccp cultivation is the contamination of samples with other mycoplasmas. Antibiotic treatment inhibits the growth of classic bacteria, but mycoplasmas such as *M. arginini* or *M. ovipneumoniae* are often isolated together with Mccp as they are natural colonisers of the upper respiratory tract of goats (Thiaucourt *et al.*, 1996).

Biochemistry:

Biochemical tests (Rosendal, 1994) cannot identify an isolate unequivocally, which at present needs to be done in combination with serological, and if possible, genetic means. These tests are, nevertheless, used as a preliminary screening system and provide supportive data for serological findings. Those most commonly used are glucose breakdown, arginine hydrolysis, 'film and spots' formation, reduction of tetrazolium chloride (aerobically and anaerobically), phosphatase activity, serum digestion and digitonin sensitivity. The first three are performed routinely in isolation and cultivation procedures. 'Film and spots' describes an apparent wrinkling of the agar surface due to the deposition of an iridescent film of lipid, together with the development of black spots within the medium in the vicinity of aging colonies. Mccp does not exhibit this biochemical characteristic. The remaining biochemical tests as well as serum digestion, which can distinguish members of the *Mycoplasma mycoides* cluster from other small ruminant mycoplasmas, requires specialised and expensive

media or reagents, placing these procedures beyond the capabilities of many developing countries.

Antigen detection:

Mycoplasmal antigens used in hyperimmune serum production are invariably contaminated with medium constituents. As a consequence, antibodies elicited by these contaminants can cause false-positive reactions in serological identification tests. This can be avoided by cross-absorption of the antiserum with the medium used to produce the antigen. The growth inhibition test (GIT) (Jones and Wood, 1988) is the simplest and most specific, but the least sensitive of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum and detects primarily surface antigens. A Mab specific for Mccp in the GIT has been produced (Rurangirwa *et al.*, 1987d). The growth precipitation test (Krogsgaard-Jensen, 1972) detects soluble cytoplasmic and extramembranous antigens released by growing cultures. These are allowed to diffuse through solid mycoplasma growth medium towards mycoplasma antiserum during growth. The direct and indirect fluorescent antibody tests (Rosendal and Black, 1972) are the most effective of the various serological methods of identification for most mycoplasmas. The indirect fluorescent antibody test (IFA) is most commonly used and is applied to unfixed colonies on agar. Interpretation of the IFA test can be difficult due to autofluorescence produced by some species and a proportion of pure culture colonies not staining with the relevant antiserum. Poor results can also be ascribed to the use of agar cultures that have been allowed to grow for too long. Since members of the *Mycoplasma mycoides* cluster share many serological characteristics, identification of isolates should be by at least two of the tests described above.

Other antigen detecting techniques include an immunobinding assay (Guerin *et al.*, 1993; Thiaucourt *et al.*, 1994) in which pleural fluid from an infected animal is blotted onto a nitrocellulose membrane and the presence of antigen detected by a rabbit antiserum. This method, however, is not specific as the rabbit antiserum used to detect Mccp cross-reacts with other members of the *mycoides* cluster. Identification of Mccp by polyacrylamide gel electrophoresis (PAGE) of mycoplasmal proteins generates

specific results provided the sample has been sufficiently preserved. Extensive experience in recognition of PAGE protein patterns is, however, necessary.

Antibody detection:

Despite their widespread use, serological tests designed for CCPP diagnosis suffer from several limitations. Acute cases of Mccp infection rarely show positive antibody titres before death (MacOwan and Minette, 1977; Muthomi and Rurangirwa, 1983), perhaps because antibodies are 'eclipsed' by circulating mycoplasma antigens (Muthomi and Rurangirwa, 1983). Seroconversion to Mccp in experimentally infected animals has been shown by complement fixation (MacOwan and Minette, 1977) and indirect haemagglutination (Muthomi and Rurangirwa, 1983) to start 7-9 days after the appearance of clinical signs, peaking between days 22 to 30 and subsequently declining rapidly (Muthomi and Rurangirwa, 1983). These observations indicate that serology should be applied on a herd rather than on individuals and that wherever possible, paired serum samples collected 3-8 weeks apart, should be examined. Still complement fixation remains the preferred method to detect antibodies and even though they vanish after three months, it has been found to be more specific, albeit less sensitive than indirect haemagglutination. Its main disadvantage is the high level of technical expertise required. In the IHA test, red blood cells (RBC) are used either fresh and tanned or treated with glutaraldehyde. The former is more sensitive, but shows greater variability between tests whereas glutaraldehyde treatment of RBCs reduces sensitivity, but produces a much more useful test as these cells remain effective for one year if kept refrigerated. Latex beads sensitized with the polysaccharide produced by Mccp and present in culture supernatant have been used in a slide agglutination test (Rurangirwa *et al.*, 1987b). This test is presently used routinely in Kenya and provides greater specificity as there is no cross-reactivity with sera against the other three principal caprine mycoplasmas, *M. mycoides* subsp. *mycoides* LC, *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum*. Antibodies specific for Mccp have also been assayed in ELISA. Two formats have been described, the most recent being a competition ELISA. It is based on the use of a Mab which competes with goat antibodies to bind to Mccp antigen that is coated onto microtitre wells, achieving high specificity with sera of CCPP infected goats (Thiaucourt *et al.*, 1994). The alternative indirect ELISA does not make use of a Mab, but rather detects antibodies to the Mccp antigen with a horseradish peroxidase

conjugated rabbit anti-goat IgG (Wamwayi *et al.*, 1989). As a consequence of the limitations and lack of validation, of most of the assays discussed, none are applicable to diagnosing individual animals and are therefore only suitable for herd diagnosis. Accordingly, serological tests need to be accompanied by the isolation of the organism to confirm the existence of Mccp or correlate with other assays performed in parallel.

Recently, the polymerase chain reaction (PCR) has radically improved the detection and identification of many microorganisms which do not grow easily *in vitro* and indeed, PCR methods have been described for detecting Mccp. Amplification of a segment of the gene that codes for the 16S rRNA (Bascuñana *et al.*, 1994) or restriction enzyme analysis of the rRNA *rrnB* operon amplicon are two PCR based methods that are currently used (Bölske *et al.* 1996). Despite the advantages of PCR this technology has not been widely applied in African countries, as economic constraints do not allow the purchase of expensive machinery and reagents required for this technology.

1.1.6 Control

High morbidity and mortality and the ease with which Mccp spreads, make measures for controlling the disease very important. Control strategies can only be defined once the prevalence and incidence of the disease is known. Due to the lack of information and the technical problems associated with economic constraints in many developing countries, CCPP control programs still need to be properly defined. Antibiotic treatment remains the most effective way of dealing with the disease. The most active antibiotics belong to the tetracycline group and macrolide family (tylosing, erythromycin or spiramycin) (Thiaucourt *et al.*, 1996). Their disadvantages are, however, their sensitivity to heat and the expense and lack of practicality due to several treatments having to be administered.

Apart from antibiotics, vaccination is also employed as a control measure. The first experimental vaccine against Mccp comprised live Mccp in high passage (MacOwan and Minette, 1978). When inoculated intratracheally it proved innocuous and protected goats against experimental challenge. More recent work has concentrated on

inactivated vaccines. The current form used in Kenya contains lyophilised Mccp suspended in saponin (Rurangirwa *et al.*, 1987a) and provides protection for over one year.

To summarise, it is important to be able to detect outbreaks of CCPP in order for control policies to be effective. The development of PCR has greatly improved direct and specific detection of Mccp, but the serological techniques used today have several limitations which need to be considered before using these to confirm outbreaks of the disease (Section 1.1.4). Despite this antibodies can be useful tools for discriminating between organisms if they recognise species specific epitopes. The identification of such antibodies would be invaluable for the diagnosis of CCPP.

1.2 Antibodies

1.2.1 Immunity

Immunity is concerned with the recognition and disposal of foreign or 'non-self' material that enters the body, usually in the form of potentially pathogenic organisms. Immune recognition and responses largely depend on interactions involving proteins. Resistance to infection may be innate/natural or acquired/adaptive. Innate immunity is mediated by cells that respond non-specifically to foreign molecules and does not improve with repeated exposure. Innate defense mechanisms rely on phagocytosis by macrophages, cell lysis by natural killer cells and antimicrobial factors such as complement, acute phase proteins, lysozyme and the interferons. In contrast, adaptive immune responses depend on lymphocytes to mediate specific recognition of foreign molecules. Following an initial encounter with a particular molecule a second or subsequent encounter results in an enhanced or modified response to that molecule. These lymphocytes synthesise surface receptors or secrete proteins that bind specifically to foreign molecules. These secreted proteins are known as antibodies (immunogobulins). Any molecule that can bind to an antibody is known as an antigen (Roitt, 1997).

Two main types of lymphocytes that are specialised in the recognition of antigens are the B cells which develop in the bone marrow and which may subsequently differentiate into antibody producing plasma cells and T cells which differentiate in the thymus. T cell functions include helping B cells to make antibody, killing virus-infected cells, regulating the level of the immune response and stimulating the microbicidal and cytotoxic activity of other immune effector cells, including macrophages. T cells only recognise antigen when it is presented to them in association with molecules encoded by the major histocompatibility complex (MHC). Communication between the cells is either by direct cell to cell contact or by cytokines and antibodies. Each cell carries a surface receptor that can recognise a particular antigen. The success of the immune system is due to the ability of these receptors to recognise an entire universe of foreign and self-antigens (Male *et al.*, 1991). This property is related to the enormous diversity of lymphocytic receptors resulting from multiple variable-germ line genes encoding immunoglobulin receptors (VH, Vκ, Vλ) or T cell receptors (Vα, Vβ, Vδ, Vγ), that are rearranged. Furthermore, the addition or depletion of nucleotides during the rearrangement of V genes and somatic mutations occurring in the rearranged V genes also contribute to the diversity of the immune repertoire (for a review, see Bona, 1996).

B cells recognise an unmodified antigen, either free in solution or on the surface of other cells. When the antigen with which a particular antibody reacts enters the system, it reacts with the B-cell receptor and activates the cell to proliferate and actively secrete antibody molecules. The responding population will expand and develop and if the antigen is encountered again, this clonal expansion and differentiation will aid in the efficacy of the secondary response. It is usually more effective than the primary immune response since a larger population of cells will react which have undergone affinity maturation due to recombination of gene segments and somatic mutation of the rearranged genes. A second type of clonal selection accounts for tolerance. Lymphocytes with surface receptors and high affinity for autoantigens are eliminated from the immune system by a process known as clonal deletion. Self-reactive clones are deleted during B cell ontogeny. This process is active throughout an organism's life, occurring in the foetal liver while the immune system is developing and primarily in the bone marrow in the case of adults. The role of the

thymus in the development of self-tolerance is evident in that T cells are negatively selected for recognition of self-MHC and positively selected for recognition of peptide in the context of MHC. Deletion effects have not only been noted for classic self-antigens, but also for foreign antigens introduced during early T cell development (Male *et al.*, 1991). Immune recognition is of paramount importance in the normal functioning of the system since lymphocytes must recognise antigens of potential pathogens while at the same time tolerating molecules of the body's own tissues.

1.2.2 Antigenicity and Epitopes

The antigenic reactivity ('antigenicity') of a protein refers to its capacity to bind specifically to the functional binding sites (or paratopes) of certain immunoglobulin molecules. Atassi (1984) suggested that each protein molecule contains only a limited number of antigenic determinants. A more widely held view indicates that almost all surface-exposed sites on a polypeptide chain are potentially antigenic and thus can be recognised by antibodies (Benjamin *et al.*, 1984). Not all antigenic sites on the surface of a protein are, however, equally effective as immunogens and exhibit immunogenicity as opposed to antigenicity, and thus have the ability to induce an immune response. Several factors contribute to the magnitude and the specificity of a humoral response to an antigen, such as the genetic background of the immunised animal, cellular factors (T cell help), self-tolerance mechanisms and the route and scheme of immunogen administration (Sercarz and Berzofsky, 1988).

An antibody molecule possesses two identical paratopes made up of six highly accessible loops of hypervariable sequence known as complementarity determining regions (CDR) (Capra and Kehoe, 1975). The CDRs are present on the end of the two variable domains of the immunoglobulin molecule and interact to varying degrees with the surface of a protein antigen. That portion of the antigen that comes into contact with the paratope of the antibody constitutes an antigenic determinant or 'epitope'. In the same way that the antibody nature of an immunoglobulin is identified only when its complementary antigen has been recognised, the epitope nature of a set of amino acids in a protein can be established only by finding an immunoglobulin able to bind to it. In general, an epitope can correspond to a size of 5-7 amino acid residues (Van Regenmortel and Daney de Marcillac, 1988) and the surface of a protein can

have a number of overlapping epitopes, thereby constituting an antigenic site (Benjamin *et al.*, 1984).

Epitopes can be divided into a number of categories, two of which are linear/continuous and conformational/discontinuous. Continuous epitopes (Atassi, 1975) are defined by peptide regions lying adjacent to each other as opposed to discontinuous epitopes (Atassi *et al.*, 1976) which comprise surface residues that come into close proximity by virtue of the folding of the polypeptide chain, but which are generally not directly linked by peptide bonds. A further two categories of epitopes which have particular relevance to the antigenic structure of viruses are the so-called cryptotopes (Jerne, 1960) and neotopes (Van Regenmortel, 1966). Cryptotopes are hidden epitopes that become expressed only after fragmentation, depolymerisation or denaturation of the antigen. Neotopes are epitopes that are specific for the quaternary structure in proteins. They arise as a result of conformational changes in the monomeric subunit of viral capsids and are induced by intersubunit bonds or by the juxtaposition of residues from neighbouring subunits.

1.2.3 Cross-reactivity and specificity

The relationship between an antibody and its antigen is not absolute. In addition to recognising the epitope against which it was elicited, an antibody will always bind to a variety of related epitopes that share some structural features with that immunogen. Antibodies are not only polyspecific, but can also be heterospecific or heteroclitic (Mäkelä, 1965; Underwood, 1985) ie. they are sometimes able to bind more strongly to other antigens than to the one against which they were raised. Owing to the cross-reactive nature of antibodies, the term specificity is used when the level of discrimination that is required in any particular case is achieved, the same antibody may be considered specific or non-specific depending on the task at hand (Van Regenmortel, 1998). It is therefore accepted that epitope-paratope interactions occur more frequently due to a cross-reactive, rather than an absolute fit.

1.2.4 Affinity and avidity

The affinity of the interaction between antigen (Ag) and antibody (Ab) is also an integral part of the question of specificity. Affinity is the strength of the interaction between the antigen and antibody and can be defined through the equilibrium constant (K_A) of the association reaction: Ab +Ag $\leftrightarrow$ AgAb. If the affinity of the antigen-antibody interaction is high the equilibrium will lie over to the right. Due to the dynamic nature of the interaction, affinity can equally be defined in terms of the dissociation constant (K_D) of the reaction. If the equilibrium lies far to the left this indicates that the interaction between the antigen and antibody is weak and the contact time between the two is short. Binding affinity depends upon the area of contact between the antibody and the epitope, the closeness of fit, conformation changes necessitated by electron-cloud overlap and the distribution of charge and hydrophobic groups (Roitt, 1997). While the term affinity describes the binding of antibody to a single antigenic determinant the term avidity, expresses the effect of binding of antigen to antibody by multiple links. This includes for instance, the heterogeneity of antibodies in a serum, which are directed against each determinant on the antigen and the heterogeneity or the multivalency of the determinants themselves (Roitt, 1997).

1.2.5 Anti-idiotype antibodies

Several classes of immunglobulins exist (IgG, IgA, IgM, IgD and IgE), each class of antibodies has distinct antigenic determinants on the constant regions of their heavy chains. Variations between classes are called isotypic variants. Genetically determined variations, however, mainly in the constant regions of antibodies of the same class, give rise to allotypic variants. In addition to these variations, individual determinants which are characteristic for each antibody, are found only in the variable region. These are found either in the hypervariable or in the framework region and determine the idiotype of the antibody. The word idiotype originates from the Greek word 'idios', and means private. The internal image concept (Jerne, 1974) proposes that idiotypes represent links between the outside world of the antigens and the inner immune repertoire. The diversity of antigen receptors is reflected in the diversity of idiotypes, which are located on various segments of the variable region of heavy and light chains

of immunoglobulins. Antibodies that are raised against idiotypes are anti-idiotype antibodies (Ab2s) and can be categorised according to which idiotope they recognize; the α-Ab2s (Jerne *et al.*, 1982) recognise idiotopes associated with the framework of the variable region of an immunoglobulin. The binding of these Ab2s to their corresponding idiotope is not inhibited by antigen, but they are able to suppress or prime the clones expressing idiotypes. α-Ab2s cannot mimic the antigen. The second category of Ab2s is the γ-Ab2s (Kohler and Bona, 1984) which recognise paratope associated idiotopes and can inhibit the binding of antigen to paratope. The inhibition is due to alteration of the three-dimensional structure of the paratope preventing the interaction with the epitope. Like the α-type, the γ-type Ab2s cannot mimic the antigen. Ab2s that recognise antigen-inhibitable idiotopes that mimic the antigen are called β-type Ab2s (Jerne *et al.*, 1982). These antibodies represent the internal image of the antigen since their idiotypes cross-react with foreign or self-epitopes. This enables one to find structures or shapes which are similar, if not identical, to the epitopes of self and nonself antigens. A special category of Ab2s, ε-type (Kohler and Bona, 1984), bind to their corresponding idiotopes, as well as to the antigen interacting with antibodies expressing the idiotopes, therefore representing a particular category of internal image because their paratopes interacts with both idiotopes and epitopes.

It is important to be able to define β-type Ab2s since they can be used as tools in a broad range of applications. The first demonstration of the existence of a β-type Ab2 came from the work of Sege and Peterson (1978). They showed that Ab2s prepared against bovine anti-insulin antibodies mimicked the action of insulin in that they were able to interact with insulin receptors on tissues and stimulate the physiological action of insulin itself. To date, the antigenic mimicry of Ab2s make them valuable as substitutes for hormones (Sege and Peterson, 1978), drugs of organic origin (Schreiber *et al.*, 1980), proteins (Sege and Pete, 1978), biologically active ligands (Marasco and Becker, 1982), self-antigens (Bhattacharya-Chatterjee *et al.*, 1988) and microbial antigens (Hohmann *et al.*, 1993). An additional application of these antibodies is their promise as alternatives to conventional vaccines. This is especially of value when the protective antigen of the infectious agent is a polysaccharide or the carbohydrate moiety of a glycoprotein.

Functional, immunochemical and structural criteria (Ertl and Bona, 1988) have been used to identify β-type Ab2s. The functional criterion is based on their ability to mimic a given antigen and therefore, to induce the synthesis of antibody specific for a nominal self or foreign antigen, whereas the capacity to inhibit the antigen-paratope association is considered to be the immunochemical criterion. Together these characteristics are best used to define antibodies raised against immunoglobulins specific for polysaccharides, lipoproteins, nucleotides or synthetic drugs. The structural criterion used to identify β-type Ab2s is based on the structural identity between an epitope of the antigen and a segment of the variable region of the β-type Ab2. This criterion is best used to define β-Ab2s, which have been raised by immunisation with antibodies specific for protein antigens (for a review, see Bona, 1996). Thus Ab2s raised against Mabs which, recognise specific epitopes on Mccp have the potential to be used as substitute epitopes which could prove useful in the diagnosis of CCPP.

1.3 Epitope libraries

1.3.1 Filamentous phage architecture

The non-lytic bacteriophages fd and M13 are flexible filaments with a diameter of about 6.5 nm and a length of approximately 900 nm to 1300 nm (Smith *et al.*, 1998). Their single-stranded circular DNA genome encodes for 10 different proteins, which are involved in replication, morphogenesis and formation of the virus coat. The phage infects F' episome-bearing bacterial cells through binding of the coat protein pIII to the tip of the F pilus, followed by internalisation of the single-stranded viral DNA (ssDNA). This plus-strand serves as template for minus-strand synthesis, resulting in a double-stranded replicative form (RF). The RF is the template for mRNA transcription, RF replication and production of ssDNA progeny. Progeny virions are assembled, not in the cytoplasm, but rather by extrusion of ssDNA through the bacterial envelope. In contrast to lytic phages they do not kill the cell or prevent division. As it emerges from the cell, the ssDNA acquires its extracellular sheath of coat proteins from the membrane. The coat consists of a tubular array of approximately 2700 to 3900 copies of pVIII molecules and four minor coat proteins (pIII, pVI, pVII and pVIIII) (Smith *et al.*, 1998). Five copies of the minor coat protein

pIII are incorporated into the trailing tip of the emerging virion. Both pIII and pVIII are synthesised with posttranslationally cleaved signal peptides and before incorporation into the virion, are anchored in the inner membrane with the N-terminal portion exposed to the periplasm. The N-terminal domain of pIII binds the F pilus, but is not required for virion assembly whereas the C-terminal domain is an integral part of the capsid structure as it is needed for morphogenesis and membrane anchorage. The C-terminal portion of pVIII appears to be inside the virion, close to the DNA, while the N terminus is exposed to the outside. About 100-300 bacteriophages are produced during a bacterial life cycle (Kay *et al.*, 1996).

1.3.2 Phage display

In 1985 George Smith (Smith, 1985) showed that foreign DNA fragments could be inserted into the gene III of a filamentous phage to create a fusion protein with the foreign sequence fused to the N-terminus of pIII. The fusion protein was incorporated into the virion, which retained infectivity and displayed the foreign peptide in a form accessible to an antibody specific for the peptide. The fusion phage could be greatly enriched relative to ordinary phage by affinity selection on immobilised antibody, a process termed panning (figure 1.3.2.1) (Parmley and Smith, 1988). Phage libraries are constructed by splicing foreign DNA inserts into the gene coding for either pIII or pVIII (figure 1.3.2.2). The peptide encoded by the insert is displayed on the virion surface fused to the coat protein and is available to bind to target receptors for which it has affinity. Since there is a physical link between phenotype (the displayed peptide) and genotype (the encoding DNA), antigenic peptides can be readily identified by sequencing the phage DNA. Peptides selected do not always bear an obvious similarity to the target molecule's amino acid sequence, but may mimic their binding properties (Scott and Smith, 1990; Fehrsen and Du Plessis, 1999). Such peptides are termed mimotopes (Geysen *et al.*, 1987). If the peptide sequence of the binding phage matches the primary structure of the antigen, then the epitope can be identified directly.

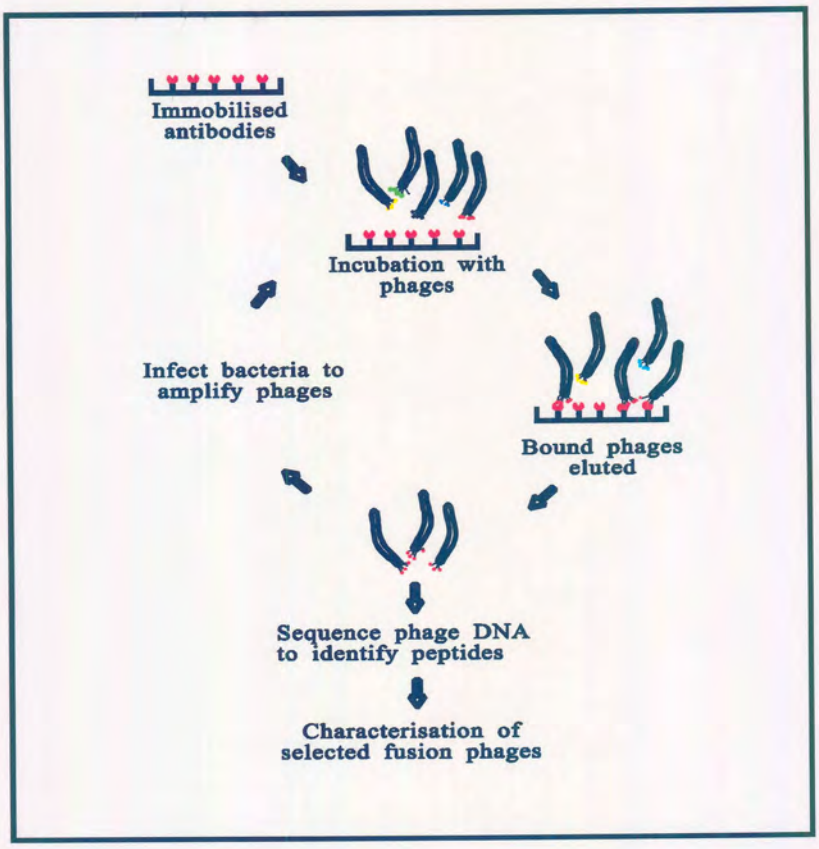


Figure 1.3.2.1

Panning. The screening process in which binding clones are separated from non-binding clones by affinity purification involves obtaining a suitable library and source of target; incubating the library with target and capturing binding phage:target complexes onto a solid phase; washing away the non-binding phage remaining in solution; eluting the binding phages from immobilised target; propagating the eluted phages; and iterating this process to enrich for binding phages. Binding phages are subsequently isolated as individual clones and then characterised using a variety of methods (diagram kindly provided by J. Fehrsen).

A variety of phage display libraries have been constructed displaying peptides and proteins with predetermined properties that can be selected and used for a variety of problems. Such libraries include peptide (Scott and Smith, 1990; Cwirla *et al.*, 1990; Devlin *et al.*, 1990); antibody fragment (McCafferty *et al.*, 1990); cDNA (Pereboeva *et al.*, 2000) and gene fragment libraries (Petersen *et al.*, 1995; Wang *et al.*, 1995).

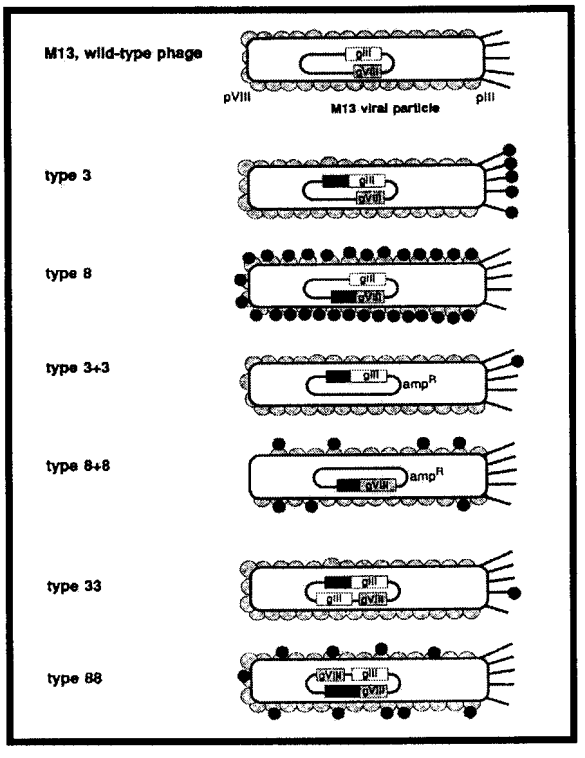


Figure 1.3.2.2

Classification of phage display vectors. Each vector system is classified as type 3 or 8 depending on whether the fusion is with proteins III or VIII. The black boxes and spheres correspond to the foreign genetic elements and their encoded peptides, respectively (Kay *et al.*, 1996).

The initial phage display libraries consisted of phages displaying random foreign peptides. These are generated by splicing degenerate synthetic oligonucleotides into a coat protein gene. The resulting libraries can be used for a variety of studies including epitope mapping (Du Plessis *et al.*, 1994; Bentley *et al.*, 2000) and the analysis of protein-protein interactions (Prezzi *et al.*, 1996). Many of these libraries make use of peptides fused to pIII, although displaying proteins as fusions to the gene VIII protein also has advantages: 1) more copies of a fusion peptide can be displayed per phage and 2) because the gene VIII protein does not participate in the absorption of phage to the pili of *E. coli*, the addition of protein domains onto the gene VIII protein does not affect phage infectivity. Peptides inserted near the N terminus of pVIII are prominently exposed on the surface of the virion and have been shown to recruit T-cell help and elicit specific B-cell antibodies without the need of adjuvants, making

display on the major coat protein (pVIII) of particular value in exploring the immune response (Greenwood *et al.*, 1991; Meola *et al.*, 1995). Limitations on the length and sequence of peptides displayed on recombinant virions (Greenwood *et al.*, 1991) is a drawback, but hybrid virions with interspersed fusion and wild-type pVIII proteins can be generated. By simultaneously expressing genes encoding the wild-type and fusion coat proteins in the same *E. coli* cell (Felici *et al.*, 1991), larger peptides can be displayed (larger than five or six amino acids). The phage display system is a powerful method for identifying epitopes and has the potential to provide novel reagents, which could be useful for the diagnosis of CCPP and other diseases. Phage displayed peptides that bind to the coat protein of cucumber mosaic virus were isolated from a random nonapeptide phage display library (Gough *et al.*, 1999). These peptides do not bind to other plant viruses and therefore have provided a cheap and convenient source of diagnostic protein. The identification of type-specific regions in glycoprotein G of the herpes simplex virus (Grabowska *et al.*, 1999) with phage display could also have application in the development of type-specific peptide based assays.

1.4 Surface plasmon resonance

1.4.1 BIACORE X system

The antigen-antibody reaction involves a series of molecular events that can be analysed by different biophysical methods to provide qualitative or quantitative data on the binding reaction. The use of biosensors to monitor interactions in real-time was first introduced in the 1990's (Jönsson *et al.*, 1991). Interaction analysis has made it possible to obtain kinetic data for a large number of protein-protein (De Haard *et al.*, 1999) and protein-peptide (Laune *et al.*, 1997) systems and has thus made it possible to select the diagnostic reagents that are most suitable for current immunoassays. Mabs intended for use in ELISA should have a reasonably fast association rate constant and a sufficiently low dissociation constant to prevent them from dissociating from the antigen during the washing step. The kinetic rate constants can be determined from a few microlitres of culture supernatant during hybridoma cell culture and clones producing unsuitable Mabs can thus be discarded at an early stage during the selection of Mab reagents using a biosensor (Karlsson *et al.*, 1991). This can also be applied to

recombinant DNA technology where changes made by site-directed mutagenesis which favorably alter the binding characteristics of a protein are quickly observed (Rauffer-Bruyère *et al.*, 1997). A biosensor may be defined as an instrument that combines a biological recognition mechanism with a sensing device. Biomolecular interaction analysis using the BIACORE X system relies on the optical phenomenon of surface plasmon resonance (SPR) (Liedberg *et al.*, 1983). SPR detects changes in the refractive index of a solution close to the surface of a sensor chip. The refractive index describes how light waves travel through a medium, the velocity of photons being different in different media. The sensor chip is a glass slide with a thin layer of gold deposited on one side, which in turn is covered with a covalently bound matrix, usually dextran, on which biomolecules can be immobilised. Monochromatic *p*-polarised light is shone through a glass prism, semi-circular in cross section, onto the flat surface of the sensor chip. The light beam interacts with the free electron constellations in the gold surface converting the incident light photons into surface plasmons, these plasmons (electron density waves) then create an electric field called the evanescent field that extends into the medium on either side of the gold film. When the analyte concentration at the sensor surface changes, there is a change in refractive index, the velocity of the plasmons is affected, and the incident light angle at which resonance occurs shift. The resonance angle is expressed in resonance units (RU) and the continuous display of RU as a function of time is presented in a sensorgram (figure 1.4.1.1). Experiments are performed under continuous, precisely controlled flow conditions using the microfluidic system for the delivery of the sample to the sensor surface. This is essential for the rapid exchange of sample and buffer at the sensor surface and accurate control of contact time to ensure results with high reproducibility (Markey, 1999).

The immobilisation of ligands to the CM5 sensor chip surface of the BIACORE system is carried out by activating the carboxylated dextran layer with a mixture of *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) to produce NHS-esters. These moieties react with primary amino-containing ligands, such as antibodies and several other proteins. Once the surface has been activated the ligand to be immobilised is diluted to an appropriate concentration in an acetate buffer of a pH lower than the pI of the ligand. This is necessary to get the ligand's net charge to positive so that the ligand is attracted to the dextran surface,

running buffer defines the baseline and all responses are expressed relative to this level.

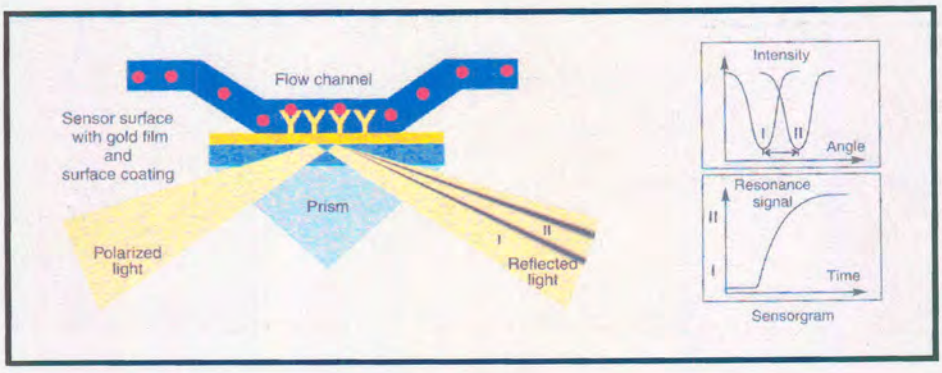


Figure 1.4.1.1

The principle of SPR. An increased sample concentration in the surface coating of the sensor chip causes a corresponding increase in refractive index which alters the angle of incidence required to create the SPR phenomenon (the SPR angle). This SPR angle is monitored as a change in the detector position for the reflected intensity dip (from I to II). By monitoring the SPR-angle as a function of time the kinetic events at the surface are displayed in a sensorgram (adapted from BIAtechnology Note 107).

Immobilisation is followed by the injection of ethanolamine, this solution has a much higher refractive index than the running buffer and the high ionic strength of this solution removes non-covalently bound material from the surface and deactivates excess reactive groups on the surface. The surface is now ready from the injection of analyte. The analyte is diluted to an appropriate concentration in running buffer in order to minimise the change in bulk refractive index. In-line reference subtraction can also be used to eliminate bulk effects. By using one of the flow cells as a control and subtracting the RU generated in the sample flow cell with the RU generated in the control cell, the bulk effect is eliminated.

When analyte is injected across a ligand surface the resulting sensorgram can be divided into three essential phases as illustrated in figure 1.4.1.2. The first phase is the association of the analyte with the ligand as the sample is injected. This is followed by an equilibrium or steady state phase, where the rate of analyte binding is balanced by

an equilibrium or steady state phase, where the rate of analyte binding is balanced by dissociation from the complex. The running buffer is injected over the surface and the third phase begins where the analyte dissociates from the ligand immobilised to the surface. The association and dissociation phases provide information on the kinetics of the analyte-ligand interaction, in other words, the rates of complex formation and dissociation. The equilibrium phase provides information on the affinity of the analyte-ligand interaction.

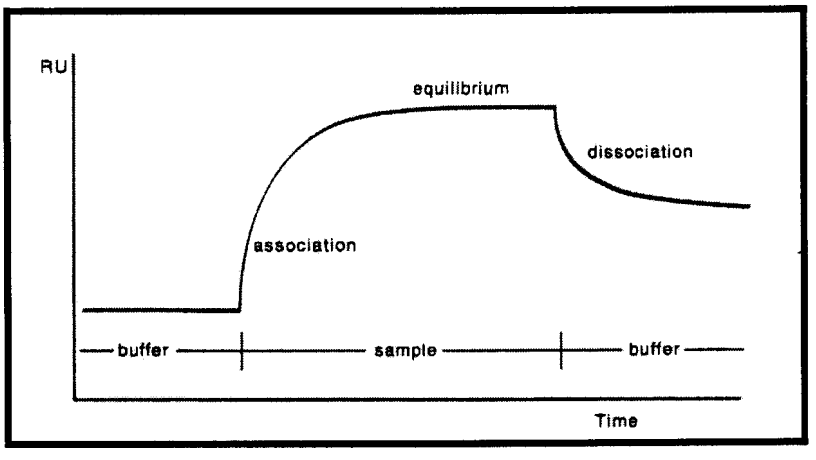


Figure 1.4.1.2
Sensorgram showing association, equilibrium and dissociation phases (adapted from BIAapplications handbook 1998).

Several sensor chips are available besides the CM5 chip, such as the C1 chip. This chip has carboxyl groups attached directly to the surface without the dextran matrix. The flat carboxylated surface may be of value when working with large analytes such as cells and virus particles. The F1 sensor chip is similar to the CM5 chip in that it also has a carboxylated surface attached to a dextran matrix, however the matrix is considerably shorter, allowing this chip to be used for analysis of larger analytes.

This technology has many advantages over the classical solid-phase assays. Conformational integrity of the immobilised protein is usually preserved on the sensor surface, none of the reactants need to be labelled and the ability to regenerate the sensor chip surface while retaining the immobilised ligand allows a series of separate

measurements to be performed with the same sensor chip. Interactions are also measured in real-time allowing kinetic rate constants and equilibrium affinity constants to be calculated. The application of this technology is far reaching, allowing for the proper design of immunoassays and for understanding the molecular basis of epitope recognition (Richalet-Sécordel *et al.*, 1996).

1.5 Motivation and aim

The goat is an important commodity in many areas of the world, where it is kept as a source of meat, milk and fiber. Often described as the "poor man's cow", it can survive in areas where cattle cannot and therefore replaces the cow in importance for a large segment of the world's population. Thus, improved goat husbandry can help maximise human food supplies from marginal agricultural lands under restrictive climatic conditions. Among the important goat diseases, mycoplasmal infections result in significant losses on the African and Asian continent.

Aspects regarding the diagnosis of CCPP have been described in the preceding review. From this it is clear that the need for a specific, sensitive and cost effective diagnostic test exists as current methods fall short of the above criteria resulting in several different tests having to be performed in order to confirm the disease. It is therefore conceivable that the generation of substitute antigens specific for Mccp could contribute to the development of improved diagnostic methods.

The aim of this study was to examine methods of generating surrogate antigens recognised by a Mccp-specific Mab, Mab 4.52 (Section 1.1.5; Thiaucourt *et al.*, 1994). Two approaches were investigated. The first exploited anti-idiotype antibody production in mice and chickens. The animals were immunised with Mab 4.52 and or the $F(ab')_2$ fragments of the antibody. The antibody was proteolytically cleaved to generate a molecule with fewer antigenic determinants in order to focus the immune response to the paratope of the antibody in some animals. The second approach used Mab 4.52 to affinity select random peptides from a XCX_{15} epitope library (Bonnycastle *et al.*, 1996). The antigenic properties of the fusion peptides and the murine and avian antibodies were characterised in a variety of immunoassay formats.

Immune recognition of the fusion peptides and the avian antibodies was further examined by surface plasmon resonance.

Chapter 2

Anti-idiotype antibodies

He that will not apply new remedies must expect new evils;
for time is the greatest innovator.
Francis Bacon, 1561-1626

Parts of this chapter have been prepared and submitted for publication:
Phage displayed peptides and anti-idiotype antibodies recognised by a monoclonal antibody directed against a diagnostic antigen of *Mycoplasma capricolum* subsp. *capripneumoniae*
Dubravka R. Benguric, Baptiste Dungu, François Thiaucourt and Dion H. du Plessis
Veterinary Microbiology

2.1 Introduction

Immune responses are shaped by several processes that promote the disposal of pathogens and hinder self recognition. Due to the enormous diversity of the immunological repertoire, the recognition of self cannot be avoided. This is the basis of the network hypothesis first described by Niels Jerne (1974) in which he postulated the coexistence of complementary partners within the antibody repertoire of an individual as well as stating that internal idiotype structures could represent the positive imprint of an antigen. An idiotope, defined as an epitope within the variable region of an immunoglobulin, is used as a structural and functional marker of the variable region. Anti-idiotype antibodies (Ab2s) can be generated by immunisation with an antibody, which contains a variety of idiotopes. The induction of an Ab2 response in syngeneic models is, however, not frequently observed (Ismaili *et al.*, 1995) and it is therefore assumed that auto-Ab2s arise mainly during an ongoing immune response. Auto-Ab2 recognise new idiotopes of somatic variants to which the immune system cannot be tolerant. For this reason, it was postulated that by immunising mice with a murine Mab directed against a *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) epitope, most of the antigenic determinants on the antibody would not be immunogenic, but that the idiotopes would be seen as foreign determinants on the Mab and an IgG response would be induced against these determinants.

A heterologous system was also investigated since the immune response in an antibody-producing animal tends to increase as its phylogenetic distance increases from the antigen source. Thus, chicken antibodies recognise more epitopes on mammalian protein as 'non-self' than the corresponding rabbit or mouse antibody (Horton *et al.*, 1984). The use of chickens as opposed to mammals for antibody production also represents both a refinement and a reduction in animal use. It is a refinement in that the collection of blood is replaced by antibody extraction from egg yolk and fewer animals are necessary since chickens produce larger amounts of antibodies than laboratory rodents. The main type of immunoglobulin isolated from egg yolk is generally referred to as "IgY", other classes are present, but in negligible amounts. Structurally, IgY is different from mammalian

IgG. The heavy chain of IgY has a molecular weight (MW) of 65kDa and is antigenically distinct from IgG since it does not have a hinge region and possesses four constant domains in addition to the variable domain (Warr *et al.*, 1995).

A Mab which recognises an epitope of Mccp and its $F(ab')_2$ fragments were used as immunogens to generate Ab2s in mice and chicken. The $F(ab')_2$ fragments were used in an attempt to focus the immune response to the variable regions of the antibody. If the resulting population of antibodies contained Ab2s directed against the combining sites of the Mab which displayed structural features in common with the epitope against which Mab 4.52 was raised, these may be suitable candidates for incorporation into an epitope-orientated assay.

2.2 Materials

2.2.1 Animals

BALB/c mice were provided by and housed at the Molecular Biology Division, Onderstepoort Veterinary Institute (O.V.I.). AVIMUNE (Lyttelton, South Africa) provided and housed the specific pathogen free (SPF) white leghorn hens.

2.2.2 Monoclonal antibodies

Ascitic fluid containing Mab 4.52, which recognises an epitope on Mccp was a generous gift from Dr. F. Thiaucourt (Thiaucourt *et al.*, 1994). A bluetongue virus specific Mab, p4.4 (Van der Walt, 1988) and an African horse sickness virus specific Mab, 1F1 (Van Wyngaardt *et al.*, 1992) were kindly provided by W. van Wyngaardt.

2.2.3 Antibodies

Goat antiserum (93.1) against Mccp was a gift from Dr. F. Thiaucourt, EMVT-CIRAD, France. Mouse IgG antibodies were purchased from Sigma and PEG precipitated anti-Mab p4.4 IgY was generously provided by W. van Wyngaardt, O.V.I. Field sera were kindly provided by the Parasitology Division, Immunology Division and by the Virology Division at O.V.I.

2.2.4 Antigen

Lysed Mccp cells were kindly provided by Dr. F. Thiaucourt, EMVT-CIRAD, France.

2.3 Methods

2.3.1 Antibody precipitation

Lyophilised ascitic fluid was reconstituted with deionised water. Antibodies were precipitated by the drop-wise addition of an equal volume of saturated ammonium sulphate. The solution was incubated on ice for 1 hour, after which the precipitate was collected by centrifugation at 10 000 g for 10 minutes. The supernatant was discarded and the pellet dissolved in 1ml of half-strength PBS (0.05 M). The solution was briefly centrifuged to collect any denatured proteins and the supernatant dialysed (10-14 kDa MW cut off) against three changes of 500 ml of half-strength PBS at 4°C (Barbara and Clark, 1982). An appropriate length of dialysis tubing was cut and soaked in deionised water to soften the membrane. The one end of the membrane was tied while the sample was injected into the open end.

2.3.2 Ion-exchange purification

Albumin was removed from the antibody solution by ion-exchange chromatography. A DE 52 cellulose (Whatman Ltd.) column was constructed by dissolving approximately 25 ml of pre-swollen resin in 200 ml of 0.1 M PBS. The resin was rinsed several times with PBS until the eluate reached pH 7.4. The column was packed with resin leaving a 1.5 cm gap between the gel-bed and the top of the column. It was then equilibrated with half-strength PBS and stored overnight at 4°C. Excess buffer was drained and 1 ml of the antibody solution was allowed to enter the gel bed, followed by 10 ml of PBS. Fractions of 1 ml were collected and the concentration (C = mg/ml) of each was determined by using the following formula: $C = A_{280nm} \times 1/1.4$. Fractions with the highest concentration were pooled, aliquoted and stored at –20°C.

2.3.3 Preparation of F(ab')$_2$ fragments

The antibody solution was dialysed against acetate buffer (0.07 M sodium acetate, 0.05 M sodium chloride pH 4) overnight at 4°C. The F(ab')$_2$ fragments were generated by incubating 45 µg of pepsin (Sigma) with every 1 mg of antibody overnight at 37 °C.

After cleavage the solution was dialysed against PBS overnight and the concentration was determined as described above (Barbara and Clark, 1982).

2.3.4 Polyacrylamide gel electrophoresis

Proteins were analysed by SDS-PAGE (Sambrook *et al.*, 1989). A 10% stacking gel was prepared by mixing 8.1 ml of distilled water, 6.7 ml of 30% acrylamide, 5 ml of 4X separating buffer, 0.2 ml of 10% ammonium persulphate and 0.008 ml of TEMED. The mixture was directly poured between assembled glass plates and allowed to polymerise. After which a 6% stacking gel solution was made with 3.1 ml of distilled water, 0.67 ml of 30% acrylamide, 0.5 ml of 4X stacking buffer, 0.04 ml of 10% ammonium persulphate and 0.004 ml of TEMED. Once the separating gel had polymerised the stacking gel was poured directly onto it and a 10-well comb placed into the stacking gel. The buffer tank of an electrophoresis apparatus was filled with 1X TGS buffer and the comb removed after polymerisation, but prior to loading the samples. Protein samples were individually mixed with an equal volume of sample buffer (2X PSB) and heated at 95°C for 5 minutes before loading. A constant voltage (120V) was applied until the bromophenol blue band had reached the bottom of the gel. Protein bands were stained with Coomassie blue for 1 hour followed by destaining the rest of the gel with destain solutions I, II and III (appendix) until the bands were clearly visible. The image was recorded using a LumiImager (Roche) after which the gel was transferred to filter paper and vacuum dried for approximately 2 hours at 80°C.

2.3.5 Indirect ELISA

Antigen was diluted to 10 µg/ml in PBS and coated onto Nunc PolySorp™ microtitre plate wells at 4°C overnight. The wells were blocked with PBS containing 2% milk powder (2% milk powder/PBS) for 1 hour at 37°C followed by three washes with PBS/0.05% Tween 20 solution. Primary antibody was diluted in 1% milk powder/PBS/0.05% Tween 20 solution and 50 µl volumes were added to the solid-phase for 1 hour at 37°C. The wells were washed three times with PBS/0.05% Tween 20 solution after which 50 µl volumes of horseradish peroxidase (HRP) conjugated immunoglobulins diluted in 1% milk powder/PBS/0.05% Tween 20 were applied to the

wells for 1 hour at 37°C. Following washing, 50 µl of 0.01 g *o*-phenylenediamine dihydrochloride (OPD) dissolved 10 ml of 0.1M citrate buffer with 5 µl of 30% hydrogen peroxide was added to each well. The reaction was stopped after a minimum period of 10 minutes by adding 50 µl of 2 N sulphuric acid and the absorbance (492 nm) measured using a BDSL Immunoskan MS ELISA reader.

2.3.6 Immunisations

Mice:

Group 1: Two female eight week old BALB/c mice were immunised intraperitoneally with 100 µg of antibody or $F(ab')_2$ fragments thereof. ISA 50 (Seppic, France) was used as adjuvant. Three and five weeks after the primary inoculation the mice were boosted with 50 µg of antibody. Pre-immunisation and two weeks after the final boost the mice were bled by nicking the tail vein with a sterilised blade. Serum was collected by centrifugation of the blood for 10 minutes at 12 000 rpm. The blood clot was kept at 4°C overnight and centrifuged again to collect any further serum.

Group 2: The same procedure was used with this group of mice, except that there were three mice in the group and all immunisations consisted of 50 µg of antibody or $F(ab')_2$ fragments. Freund's complete and incomplete adjuvant (Labretoria) was used.

Chicken:

Two white leghorn SPF hens were immunised in the breast muscle with 100 µg of $F(ab')_2$ fragments and a third hen with 100 µg of antibody on day 1. ISA 50 (Seppic, France) was used as adjuvant. Two boosts followed five and seven weeks after day 1 of 50 µg of the appropriate antibody. Eggs were collected every second day and stored at 4°C.

2.3.7 Inhibition ELISA

An inhibition ELISA was performed essentially as described in 2.3.5 with the exception that a Mab containing solution was mixed with equal volumes of a polyclonal antibody preparation and incubated at room temperature for one hour. The mixture was then allowed to interact with the solid-phase antigens for a further one hour at 37°C. HRP-

conjugated antibodies were used to detect the binding. Percentage inhibition (P.I.) was calculated using the formula: P.I. = $A_0 - A_{SAMPLE} / A_0 - A_{100}$ X 100

A_{100} = 100% inhibition (control well, background signal)

A_0 = 0% inhibition (control well, full signal).

2.3.8 IgY precipitation

The egg yolk from the eggs of the hens was separated from the white and carefully washed with deionised water. The yolk was dispersed in four volumes (yolk volume) of 0.1 M PBS pH 7.6. The yolk lipids were precipitated by dissolving 3.5% PEG M_r 8000 in the mixture and centrifuged for 20 minutes at 5000 g. The precipitate was discarded and the supernatant decanted through a pre-wetted pad of layered cotton wool. Eight and a half percent PEG was added to the filtrated supernatant and dissolved. After centrifugation at 5000 g for 25 minutes the pellet was dissolved in 2.5 volumes of PBS. Next, the PEG content was increased to 12% to precipitate the IgY. The pellet was re-suspended in 0.25 volumes of PBS and the concentration (C) was determined by using the following formula: C = A_{280nm} X 1/1.35. The preparation was aliquoted and stored at 4°C (Polson *et al.*, 1985).

2.3.9 Immunoaffinity purification

Immunoaffinity purification was done at room temperature. An AminoLink® Plus column (PIERCE) was equilibrated with PBS or coupling buffer (PIERCE) and 2 ml (1 mg/ml) of the ligand to be coupled to the column was added to the gel slurry followed by 40 µl of reducing agent (PIERCE). The column was capped and mixed gently overnight for coupling. The coupling buffer was drained and the gel was washed with 5 ml of coupling buffer or PBS. The remaining active sites were blocked by first washing the gel with 4 ml of quenching buffer (PIERCE) after which 2 ml of quenching buffer and 40 µl of reducing agent were incubated in the reaction slurry for 30 minutes with gentle mixing. Next, the column was washed with four volumes of 5ml of AminoLink® wash solution. Eluate was monitored for unbound ligand. After which the column was rinsed with two volumes of 5 ml of PBS containing 0.02% NaN_3 (PBS/0.02% NaN_3). The bottom of the column was capped and 5 ml of PBS/0.02% NaN_3 was applied followed by

pushing a polyethylene porous disc 1 mm into the gel surface using an inverted serum separator. After the bottom cap was removed and the buffer reached the top porous disc, the flow stopped automatically and the column was capped again and stored at 4°C overnight.

The column was treated before each affinity purification by draining the excess buffer and equilibrating the column with 8 ml of coupling buffer or PBS. A volume of 1 ml of sample was applied to the column and allowed to enter the gel bed followed by 200 µl of PBS to rinse the disc. A volume of 2 ml of PBS was added to the column and incubated for 1 hour. The caps were removed and the eluate collected and the protein concentration was calculated ($A_{280\ nm}$). The column was washed with 14 ml of PBS after which bound proteins were eluted with 0.1 M glycine pH 2.5 or 0.1 M triethylamine solution pH 12. When bound proteins were collected each 1 ml fraction was neutralised with 1 M Tris pH 9.1 or 1 M Tris pH 7.4 respectively. Before storing the column at 4°C residual protein was stripped off the column with 1 M NaCl and equilibrated with 8 ml of PBS/0.02% NaN_3.

2.3.10 Surface plasmon resonance

An F1 Pioneer chip (Biacore AB, Sweden) was docked and primed in the BIACORE® X system. All experiments were performed at 25°C. A single mode of detection was chosen over flow path 2 at a flow rate of 5 µl/min. Ligand (50 µg/ml) was diluted in acetate buffer of a pH unit lower than the pI of the ligand for immobilisation via the amine-coupling procedure. The ligand acquires a net positive charge and is attracted to the dextran matrix, which has a net negative charge. The chip surface was activated by loading the sample loop and injecting 35 µl of a mixture of NHS and EDC, followed by 35 µl of the ligand solution. A volume of 35 µl of 1 M ethanolamine hydrochloride pH 8.5 was injected to deactivate excess reactive groups once the coupling procedure was complete. This was followed by an additional pulse of 5 µl of deactivation solution to condition the surface before performing a run. Ligand to be used as a control was immobilised in flow cell 1 using the same procedure. Samples containing analyte were diluted (50-100 µg/ml) in HBS buffer (Biacore AB, Sweden) and 30-50 µl volumes were

injected over the sensor chip surface at a flow rate of 10 µl/min. The surface was regenerated after each injection with 10 µl of 0.1 M glycine pH 2.5 (Biacore AB, Sweden) or 0.1 M triethylamine solution pH 12.

2.3.11 BIO-RAD protein assay

Fractions containing protein were identified using the Bio-Rad protein assay dye reagent concentrate. Eight microlitres of each sample was dotted onto a piece of parafilm (Whatman Ltd.) to which 2 µl of the dye concentrate was added. Each drop that changed to a blue colour contained protein. The corresponding samples were pooled and the absorbance measured ($A_{280\ nm}$) using an UNICAM HEλIOS UV-Vis spectrometer after which the protein concentration was calculated as in 2.3.8.

2.3.12 Concentration of proteins

The protein content of some of the pooled samples had to be concentrated to at least 50 µg/ml for further experiments. A volume of 1ml was injected into a PIERCE slide-a-lyzer mini dialysis unit with a molecular cut off value of 3500 Da. After dialysis the protein was concentrated by placing PEG 8000 onto the dialysis unit and monitoring the absorption of water by the polymer. The absorbance ($A_{280\ nm}$) was measured and the protein concentration was calculated as in 2.3.8.

2.4 Results

2.4.1 BALB/c mice

Purified Mab 4.52 was necessary for all the approaches used to generate Ab2s. The lyophilised Mab was reconstituted in deionised water and precipitated with saturated ammonium sulphate solution. An additional purification step was performed to remove serum albumin. For this an anion exchange was constructed using diethylaminoethyl cellulose. This material is suitable for the chromatography of proteins with isoelectric point (pI) values less than 7. Once applied to the column the antibodies move through the column while the albumin with a net negative charge was bound by the anion exchange.

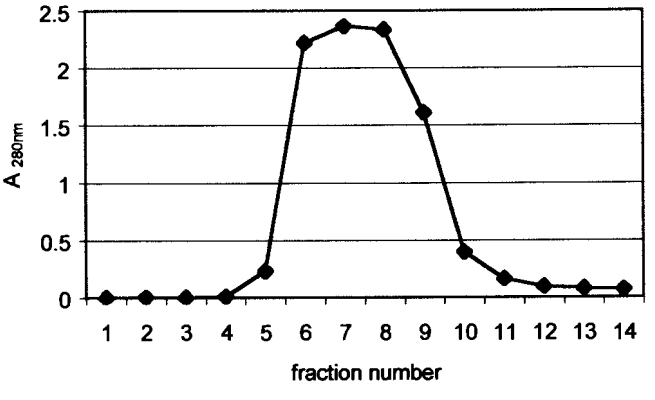


Figure 2.4.1

Absorbance (280 nm) values of 1 ml fractions collected after ion-exchange chromatography of Mab 4.52 on a DE 52 cellulose column.

Figure 2.4.1 shows the absorbance values of each 1ml fraction collected. Fractions 5 to 10 were pooled and the IgG concentration was determined spectrophotometrically to be 1.5 mg/ml. In order to show that the pooled fractions were not contaminated with any unwanted proteins, approximately 5 µg of IgG_{2A} was separated by SDS-PAGE and the proteins stained with Coomassie blue (figure 2.4.2). Analysis of the IgG preparation after electrophoresis yielded two bands: one with a MW of 55 kDa and another of 25 kDa.

These bands are characteristic of the heavy and light chains visible after reduction of the disulphide bonds of IgG. No other bands were visible.

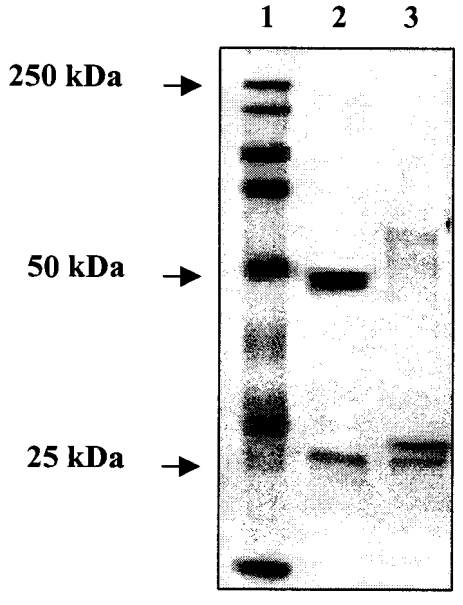


Figure 2.4.2

10% SDS-PAGE analysis of antibody and antibody fragments. Mab 4.52 (lane 2) and F(ab')$_2$ fragments of Mab 4.52 (lane 3) stained with Coomassie blue. Lane 1 shows the protein MW markers (Rainbow markers, AEC-Amersham). The samples were treated with reducing sample buffer and electrophoresed.

In order to reduce the number of antigenic determinants against which the rodents could respond and so focus the response to the variable determinants on the Mab, the IgG preparation was proteolytically cleaved with pepsin. This enzyme partially fragments antibodies releasing the antigen binding domains still bound together by a disulphide bond, a fragment known as F(ab')$_2$. Dialysis against PBS removed the Fc fragments and deactivated the enzyme with the change in pH. The F(ab')$_2$ fragments were also analysed by SDS-PAGE (Figure 2.3.2). Under reducing conditions, the F(ab')$_2$ fragments yielded two bands of approximately 25kDa. The concentration of the F(ab')$_2$ fragment preparation was determined to be 747 µg/ml.

To ascertain whether the pepsin treatment was successful an ELISA was performed. The $F(ab')_2$ fragments were coated onto microtitre plate wells and HRP conjugated rec-protein A was added. Since protein A binds to the Fc region of immunoglobulins, the absence of a signal indicated that the digestion was satisfactory (figure 2.4.3).

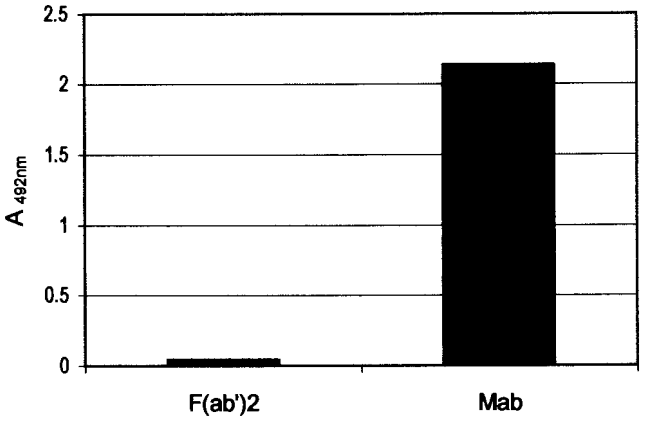


Figure 2.4.3

Reactivity in indirect ELISA of HRP-conjugated rec-protein A (1:2000, Zymed) with immobilised Mab 4.52 (10 µg/ml) and $F(ab')_2$ fragments (10 µg/ml).

Mab 4.52 reacts with a Mccp protein, the characteristics of which are unpublished. The Mab and $F(ab')_2$ fragments were individually used as immunogens to generate Ab2s in mice (2.3.6). Serum was collected 14 days after the second boost from each mouse and analysed for Ab2 response by an indirect ELISA. The mice of group 1 showed no significant response to the immunisations in relation to non-immune serum (figure 2.4.4).

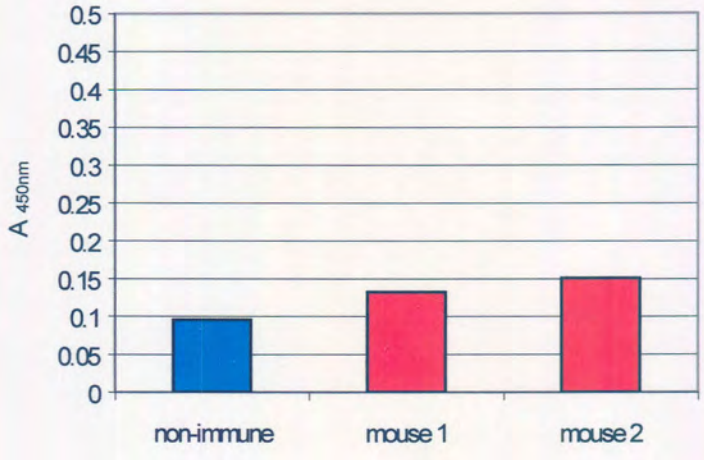
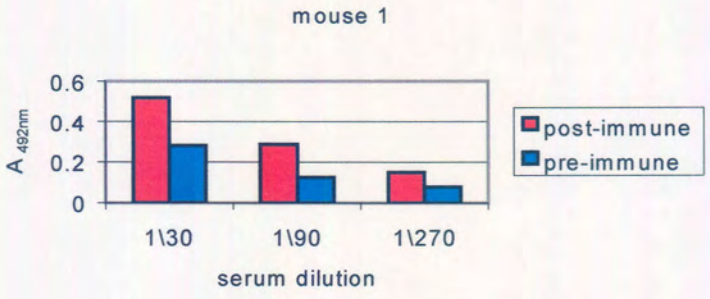
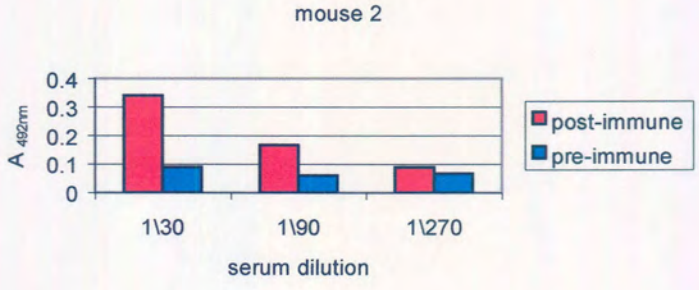


Figure 2.4.4

Binding in ELISA of immunoglobulins in murine sera (1:20) to immobilized $F(ab')_2$ fragments (10 µg/ml), seven weeks after primary inoculation. Mouse 1 was immunised with Mab 4.52 and mouse 2 with its $F(ab')_2$ fragments. ISA 50 was used as adjuvant.

The mice in group 2, however, showed a 50% or higher signal than pre-immune serum at a dilution of 1:30 and 1:90 (figure 2.4.5). The sera were also tested in an inhibition ELISA with Mab 4.52 and mycoplasma cell lysate. To determine whether there were Ab2s in the sera that recognise and bind the idiotope, which is located within or near the antigen-combining sites of Mab 4.52, each mouse serum was incubated with the Mab before adding this solution to cell lysate-coated microtitre plate wells. No inhibition was observed with sera from any of the three mice (group 2) used in the experiments (figure 2.4.6).





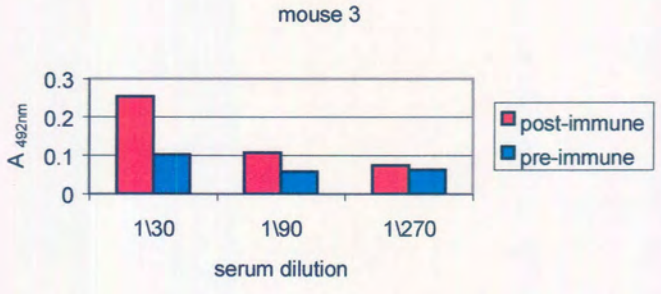
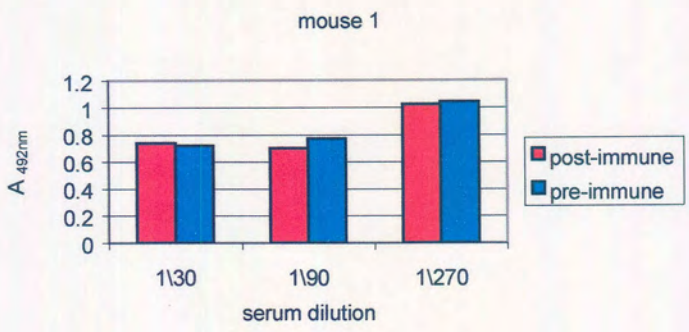
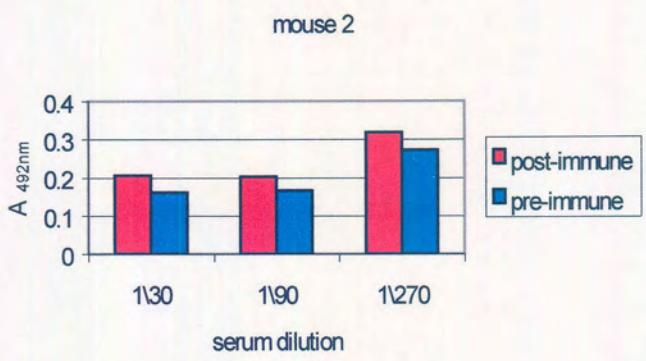


Figure 2.4.5

Indirect ELISA of pre- and post-immune serum from mice in group 2 with $F(ab')_2$ fragment (10 µg/ml) coated microtitre plate wells. Mouse 1 and 2 were immunised with Mab 4.52 and mouse 3 with $F(ab')_2$ fragments. Freund's complete and incomplete adjuvant was used.





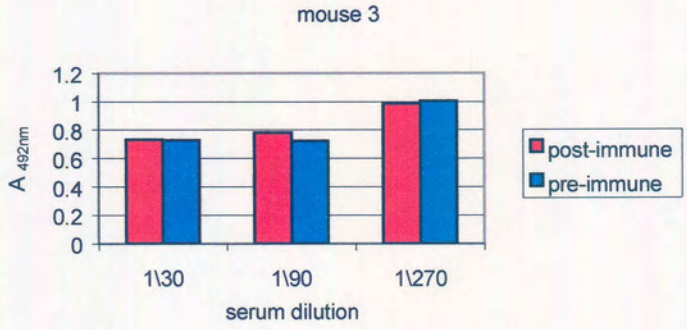


Figure 2.4.6

Competition ELISA showing inhibition of the binding of Mab 4.52 to lysed Mccp antigen (1:2000) by post-immune serum from group 2 mice. Each dilution was assayed in duplicate and averages are shown. Pre-immune serum from each mouse was used as a control and HRP conjugated rabbit anti-mouse immunoglobulins (1:2000, Dako) detected binding.

Mab p4.4 is an IgG_{2A} antibody, the same isotype as Mab 4.52. Serum from mouse 2 of group 2 was cross-absorbed with ascitic fluid containing Mab p4.4 to remove any anti-isotype antibodies in the serum and applied to $F(ab')_2$ fragment coated wells. Comparison of the signals achieved in the indirect ELISA (figure 2.4.5) at a dilution of 1:30 and 1:90, with the signals of the ELISA shown in figure 2.4.7, a drop of 75% and 64% respectively was obtained. The signals in this ELISA are comparable with the background value expected due to non-specific adsorption of proteins to the plastic microtitre well surface. It is apparent that the mice were not suitable producers of Ab2s with the methods described.

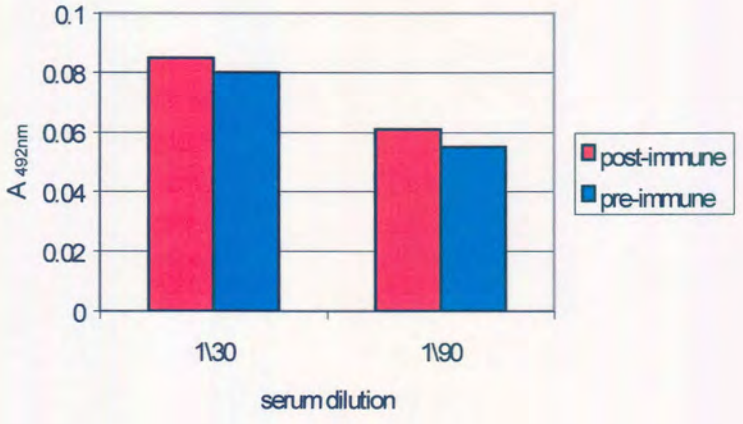


Figure 2.4.7

ELISA showing reactivity of cross-absorbed pre- and post-immune serum of mouse 2 of group 2 with $F(ab')_2$ fragment (10 µg/ml) coated microtitre plate wells. HRP conjugated rec-protein A antibodies (1:2000, Zymed) detected any binding.

2.4.2 SPF chicken

The use of chickens as Ab2 producers was also investigated, as this species should mount an immune response to the entire Mab. Chickens are also more convenient as large quantities of antibodies can be isolated from the egg yolk.

Ab2s to Mab 4.52 were raised in laying hens by immunising two birds with $F(ab')_2$ fragments derived from the Mab and one with the entire Mab 4.52. After approximately eight weeks, immune egg-yolk IgY was extracted from the eggs by PEG precipitation (section 2.3.8). The IgY from the hens immunised with the $F(ab')_2$ fragments was pooled and its concentration was determined. To ascertain whether the preparation contained Ab2s, its ability to inhibit the interaction of Mab 4.52 with the original antigen in the form of an immobilised lysate of Mccp was examined in ELISA (section 2.3.7). Figure 2.4.8 shows that the IgY was indeed an efficient inhibitor and could completely inhibit binding of Mab 4.52 to the lysate when used at a concentration of 500 µg/ml.

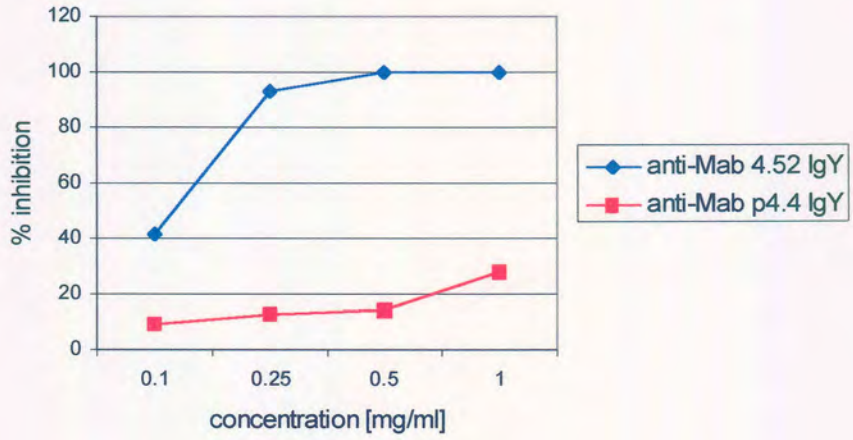


Figure 2.4.8

Inhibition of binding of Mab 4.52 (1 µg/ml) to Mccp lysate (1:2000) adsorbed to microtitre plate wells by IgY antibodies made against Mab 4.52 (◆). Percentage inhibition was calculated relative to binding in the absence of the Mab. IgY antibodies made against Mab p4.4 were used as control (■). Binding was detected with HRP conjugated rabbit anti-mouse antibodies (1:2000, Dako).

To prevent possible cross-reactions between the anti-Mab 4.52 IgY and conjugated mammalian antibodies in ELISA, the preparation was depleted of antibodies specific for murine allotype and isotype determinants by passing it over an immunoaffinity column of immobilised murine IgG (section 2.3.9). Approximately 1mg/ml of mouse IgG was covalently coupled to a 4% beaded agarose support by activating the matrix with sodium

cyanoborohydride solution to form aldehyde functional groups. These functional groups react with the amines present on the antibodies to form a stable covalent linkage.

An indirect ELISA was used to determine whether the IgY preparation had been sufficiently depleted of anti-mouse antibody activity. Either Mab p4.4 was coated onto microtitre plate wells and the 'depleted' IgY added or rec-protein A was coated onto wells to capture antibodies present in non-immune mouse serum followed by the addition of the treated IgY and the untreated IgY as a control. Figure 2.4.9 shows that both ELISAs could be used to monitor the efficiency of immunoaffinity purification.

A: **B:**

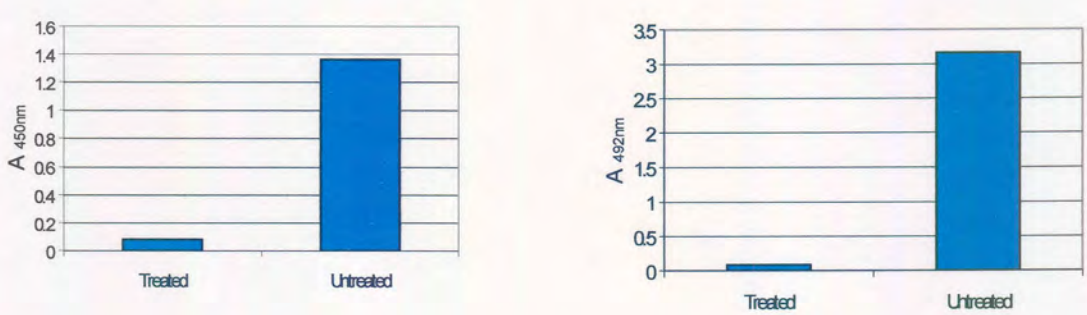


Figure 2.4.9

Indirect ELISAs used to monitor immunoaffinity purification efficiency. Antibodies in non-immune mouse serum (1:100) were captured by rec-protein A (10 μg/ml, Zymed) coated onto microtitre plate wells (A). Mab p4.4 (10 μg/ml) was also coated onto microtitre plate wells (B) and treated IgY (20 μg/ml) allowed to react. Untreated IgY was used as a control and sheep anti-chicken HRP-conjugated antibodies (1:5000, The Binding Site) detected any binding.

When the 'depleted' IgY preparation was adsorbed to microtitre plate wells, followed by the addition of Mab 4.52, the Mab was not able to detect anti-idiotype activity, however,

when the Mab was immobilised and the 'depleted' IgY added, a signal of 3.023 was obtained (figure 2.4.10). The ELISA results were confirmed by Biacore analysis. The Mab was immobilised (section 2.3.10) onto a F1 sensor chip (figure 2.4.11) and the binding curves of various concentrations of 'depleted' IgY injected over the surface immobilised with 5300 resonance units (RU) of Mab 4.52 are shown in figure 2.4.11. As the concentration of 'depleted' IgY increased so did the relative binding rate. At a concentration of 100 μg/ml of 'depleted' IgY a maximum response of 57 RU was recorded.

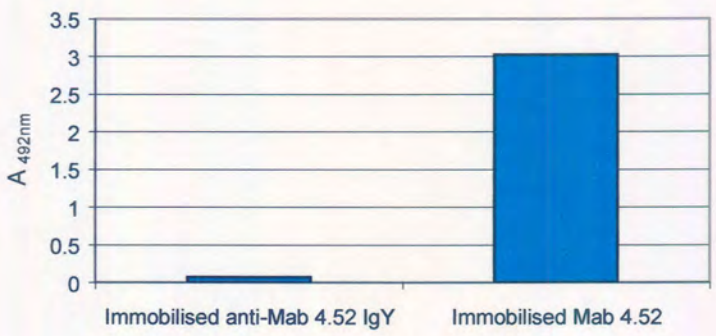


Figure 2.4.10

Indirect ELISA for detection of anti-idiotype IgY. The bar on the left-hand side of the diagram shows the signal when treated IgY made against Mab 4.52 was coated (10 μg/ml) onto microtitre plate wells, followed by the addition of Mab 4.52 (10 μg/ml). The bar on the right-hand side of the diagram depicts the signal generated when Mab 4.52 was coated (10 μg/ml) and the treated IgY added. Antibodies that bound to immobilised IgY were detected by HRP conjugated rabbit anti-mouse antibodies (1:2000, Dako) and those that bound to Mab 4.52 were detected by HRP conjugated sheep anti-chicken antibodies (1:5000, The Binding Site).

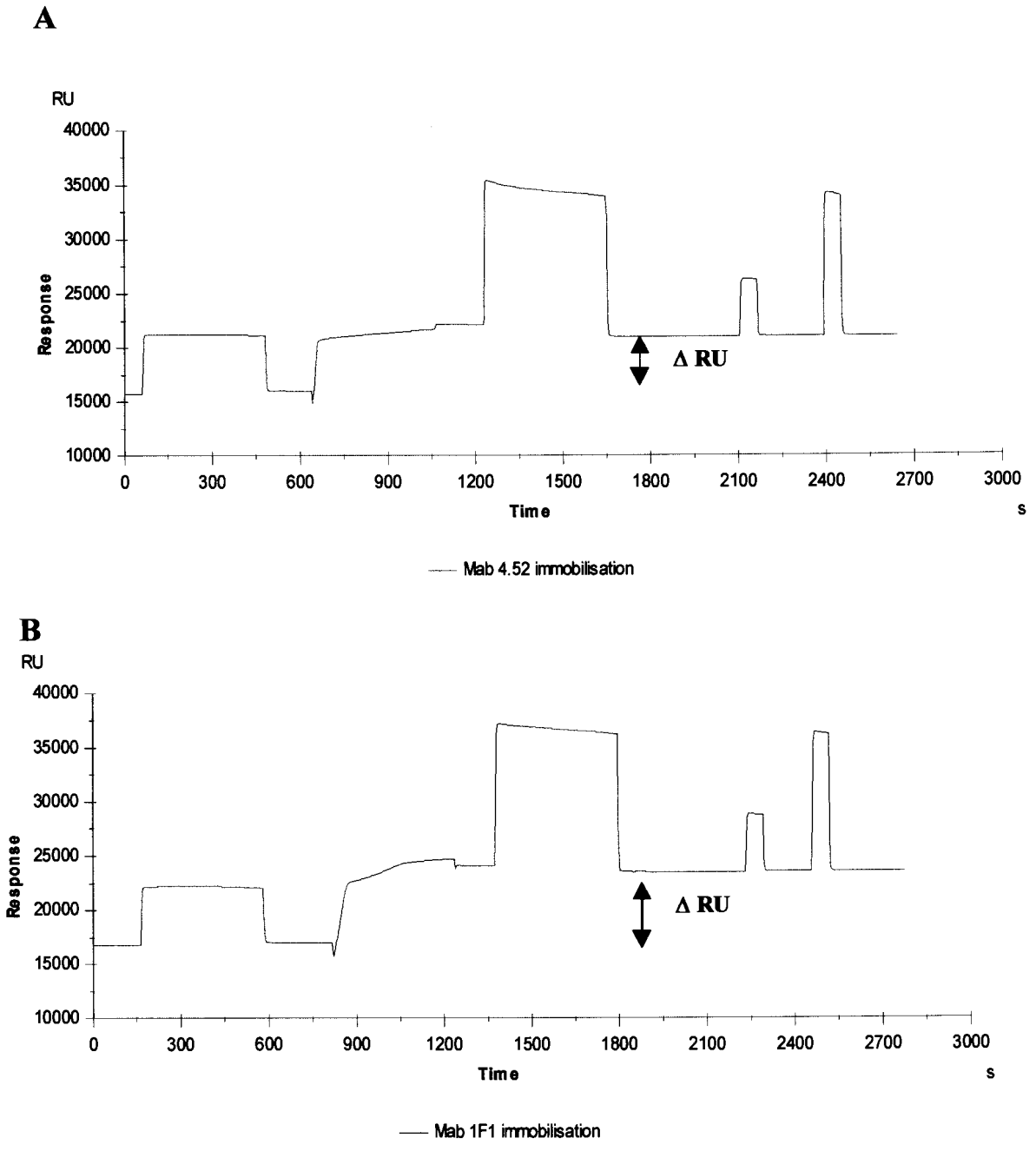


Figure 2.4.11

Sensorgrams showing the immobilisation of 50 µg/ml of Mab 4.52 (A) and Mab 1F1 (B), diluted in acetate buffer pH5, via primary amine coupling to the dextran surface of a F1 sensor chip. ΔRU represents the degree of immobilisation achieved in each case.

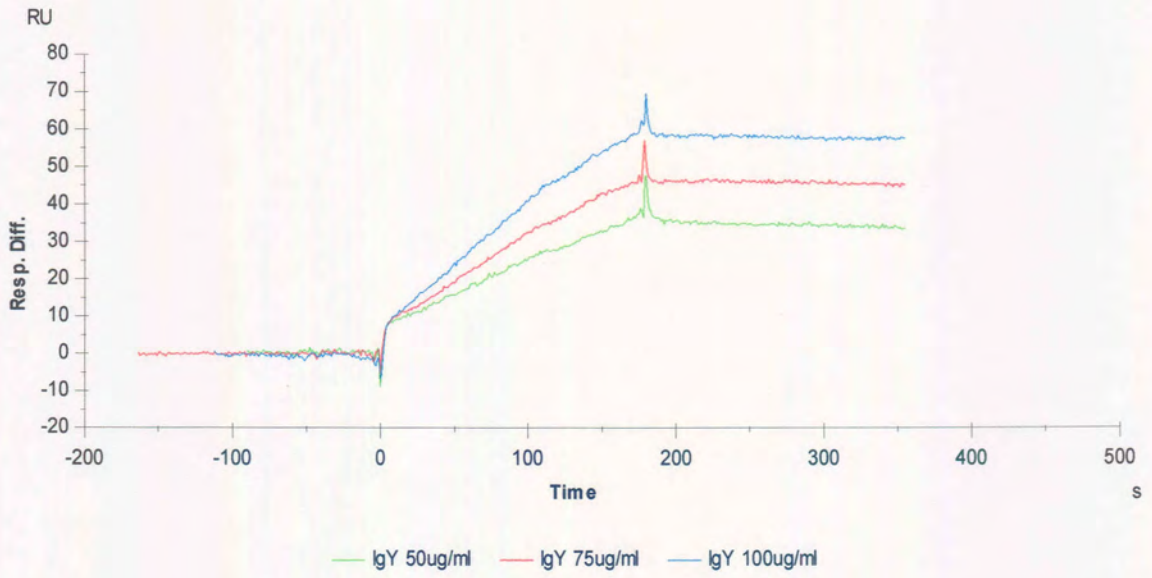


Figure 2.4.12

Overlay plot illustrating the binding of treated anti-Mab 4.52 IgY at various concentrations to immobilised Mab 4.52 (5300 RU). A volume of 30 µl of 'depleted' IgY was injected at a flow rate of 10 µl/min. The responses shown were reference subtracted with the control flow cell, which had an irrelevant Mab immobilised (6700 RU) to the dextran matrix. Dissociation of bound IgY started at 180 seconds.

When immobilised onto microtitre wells the epitope density of the Ab2s was probably too low to be detectable. For this reason the IgY preparation was immunoaffinity purified on a column to which Mab 4.52 was immobilised. The idea was to capture all the antibodies in the IgY population that recognise determinants on the Mab and so increase the epitope density of the Ab2s. Using the same procedure as when the mouse IgG was immobilised, 1 mg/ml of Mab 4.52 was covalently bound to the agarose matrix. Antibodies specific for Mab 4.52 remained bound to the immobilised Mab whereas the remainder were collected in the eluate. Antibodies specific for Mab 4.52 were released from the matrix at either high or low pH. Each fraction was neutralised accordingly and tested for Ab2 activity in ELISA (section 2.3.5), by the BIO-RAD protein assay (section 2.3.11) or by surface plasmon resonance (section 2.3.10). The fractions that produced an ELISA signal (figure 2.4.13), colour change in the protein assay (not shown) or a response in the Biacore (figure 2.4.14) were pooled and their concentration was determined.

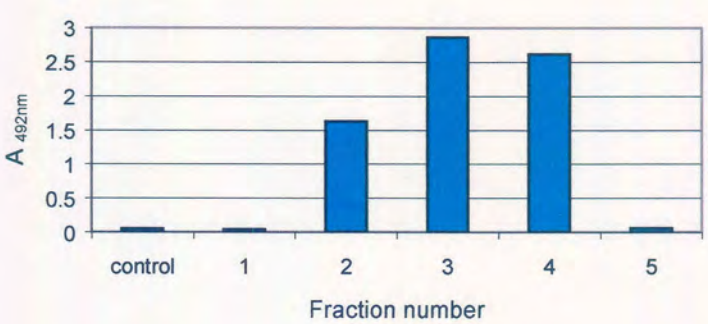


Figure 2.4.13

Monitoring of affinity purified IgY fractions by ELISA. A volume of 50 µl of each affinity purified fraction (50 µg/ml) collected was coated onto microtitre plate wells and allowed to react with Mab 4.52 (10 µg/ml). HRP conjugated rabbit anti-mouse antibodies (1:2000, Dako) detected binding. IgY that had not been affinity purified was used as a control.

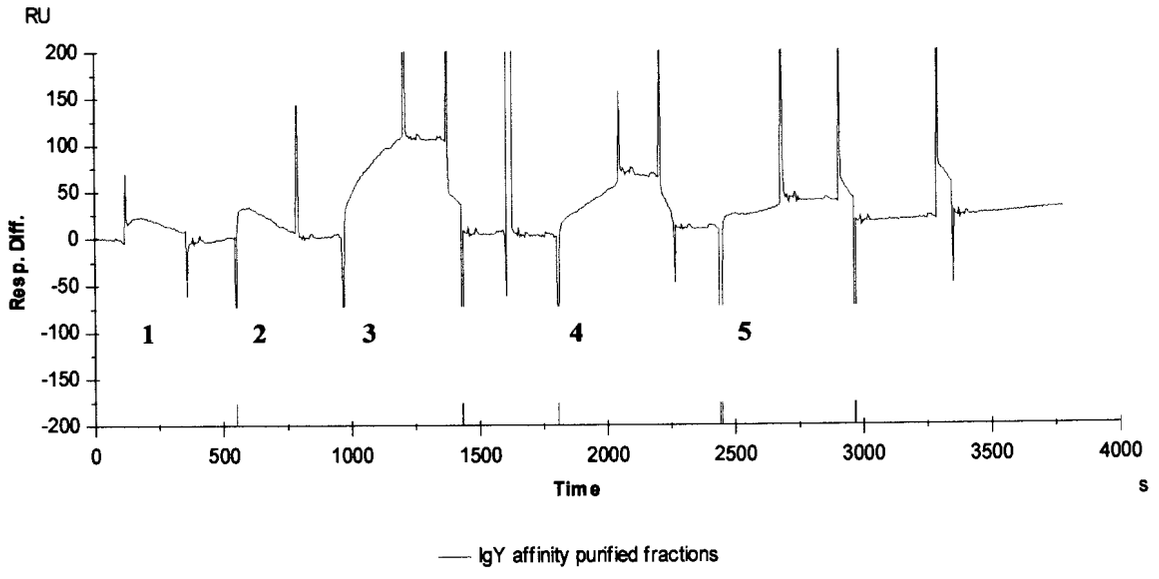


Figure 2.4.14

Sensorgram showing the binding of IgY in each affinity purified fraction (numbered 1-5). A volume of 40 µl was injected over the Mab 4.52 immobilised F1 chip at a flow rate of 10 µl/min. The response level of each fraction was subtracted from the total response obtained during sample injection, so that the response illustrated is the response related to bound antibody. The surface was regenerated between each injection with 10 µl of 0.1 M triethylamine solution.

The pooled affinity purified IgY fractions were coated at 10 µg/ml onto microtitre plate wells and Mab 4.52 was added at various concentrations. The resulting titration curve is shown in figure 2.4.15, this indirect ELISA shows that by affinity purifying the IgY on the Mab the epitope density of the Ab2s was increased and they could be detected in an ELISA. The ELISA results were confirmed by Biacore analysis. Affinity purified IgY was dialysed against three changes of PBS and the volume of the preparation was reduced, to increase the concentration of protein, by placing PEG 8000 onto the dialysis unit and monitoring the absorption of water by the polymer. The IgY was then diluted to 50 µg/ml in acetate buffer pH 4 and approximately 200 RU was immobilised onto a CM5 sensor chip by activating the carboxylated dextran matrix (Figure 2.4.16).

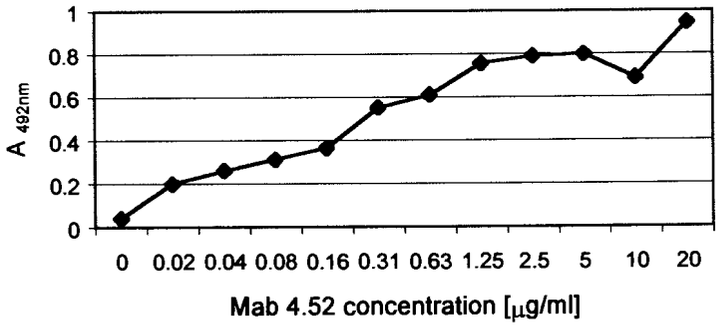


Figure 2.4.15

Titration curve of Mab 4.52 binding to affinity purified IgY coated (10 µg/ml) onto microtitre plate wells. Each data point represents the average reading from duplicate wells. HRP conjugated rabbit anti-mouse antibodies (1:2000, Dako) detected binding.

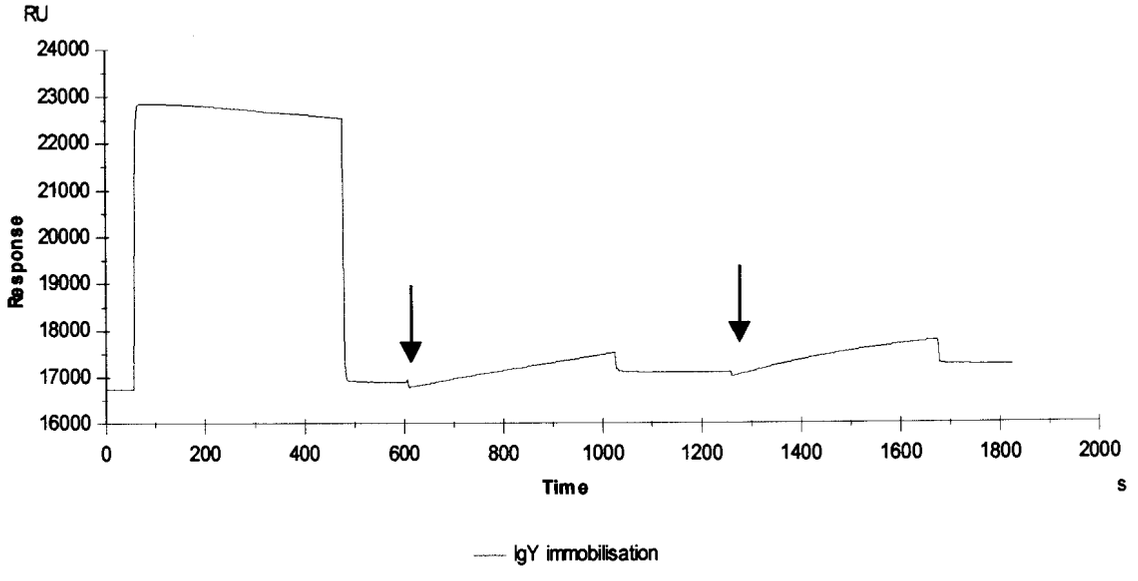


Figure 2.4.16

Sensorgram showing the immobilisation of 50 µg/ml of affinity purified IgY showing anti-idiotype activity by ELISA. IgY was injected at 5 µl/min over the activated dextran surface twice to increase the antibody immobilisation level as indicated by the arrows. Flow cell 1 was left uncoupled and served as a reference cell.

Mab 4.52 was then injected over the sensor chip surface at time 0 and a signal of 34 RU at 50 µg/ml of Mab 4.52 was recorded (figure 2.4.17). When the same concentration of an irrelevant Mab, Mab1F1, was injected over the chip surface no binding was apparent.

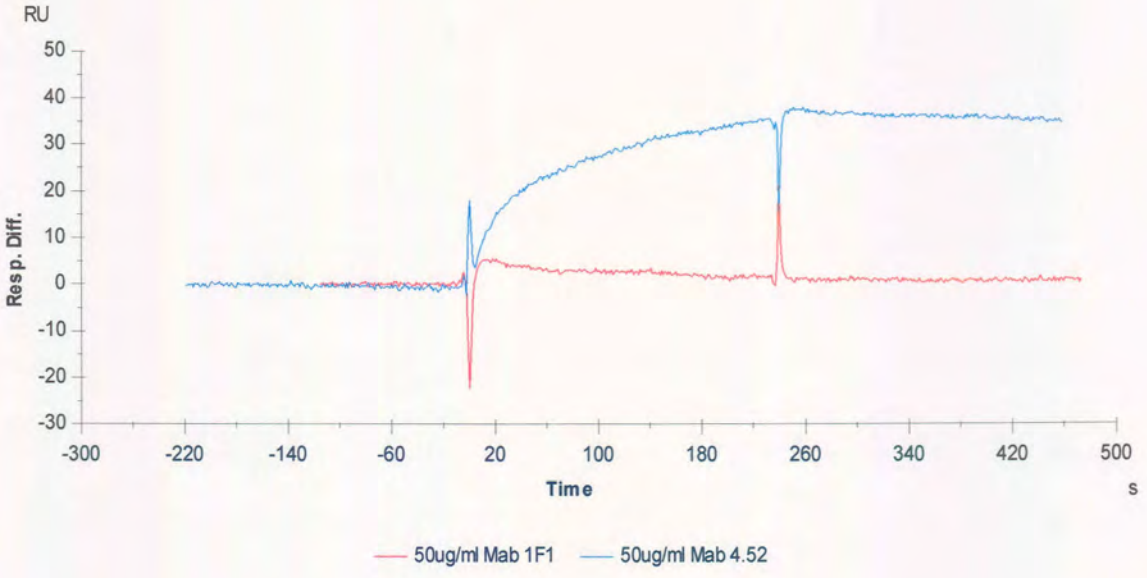


Figure 2.4.17

Overlay plot of two binding curves illustrating the specificity of the affinity purified IgY immobilised to the CM5 sensor chip. Mabs were diluted to 50 µg/ml in HBS buffer and injected over the surface at a flow rate of 10 µl/min. At 240 sec injection is stopped and the antibodies start to dissociate from the immobilised IgY.

The dissociation of Mab 4.52 from immobilised affinity purified IgY was very slow indicating a strong affinity of the Mab for the IgY. In a further experiment, the reciprocal of the one just mentioned, the affinity purified IgY was injected over immobilised Mab 4.52 and a response of approximately 50 RU was recorded (figure 2.4.18). The concentration of IgY injected over the sensor chip was not determined, as the protein

content was too low for accurate measurement by a spectrophotometer. Therefore, only two samples of 50 µl each were injected. The dissociation of the affinity-purified IgY from the immobilised Mab was also very slow, possibly the effect of avidity due to the polyclonal nature of the immunoglobulins.

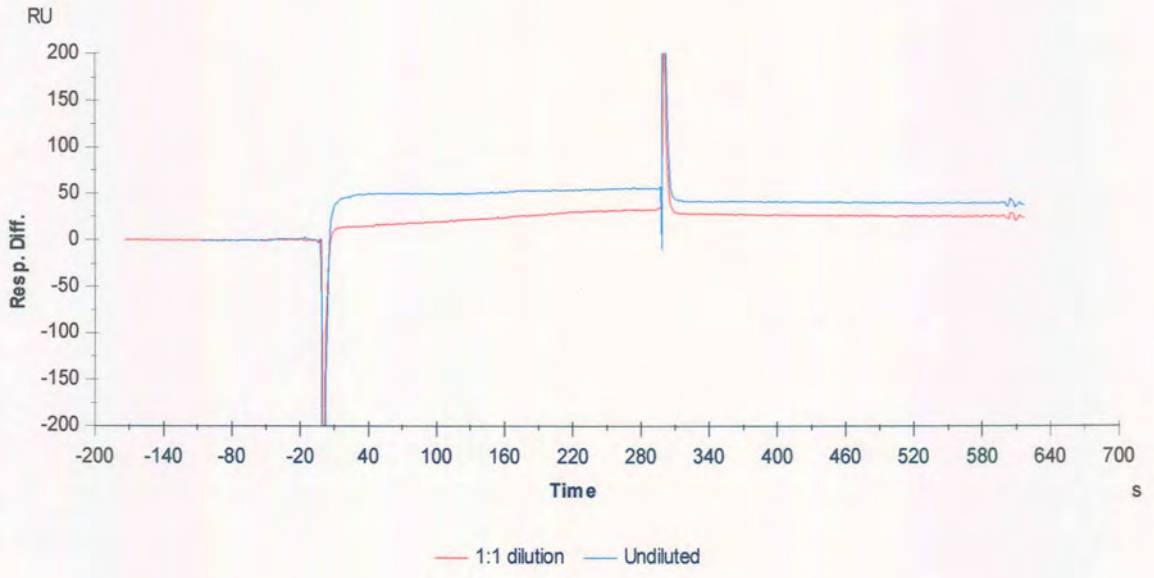


Figure 2.4.18

Sensorgram of affinity purified anti-Mab 4.52 IgY binding to immobilised Mab 4.52. The IgY was injected undiluted as well as diluted 1:1 in HBS buffer at a flow rate of 10 µl/min. After each injection the flow cells were washed with buffer and bound antibody removed by injecting 0.1 M triethylamine solution pH 12 for 1 minute.

Despite being recognised by Mab 4.52 as antigen in indirect (figure 2.4.15) and inhibition ELISA (figure 2.4.8) and by surface plasmon resonance (figure 2.4.17), a goat antiserum (93.1) directed against inactivated Mccp cell lysate did not specifically bind to immobilised affinity purified IgY in ELISA (figure 2.4.19). Similarly, no evidence of binding could be shown by surface plasmon resonance (figure 2.4.20). In ELISA, at the lowest dilution (1:25), the positive serum reacted more strongly with immobilised affinity

purified IgY than the negative serum. Pre-immune IgY was also immobilised onto microtitre plate wells and the positive and negative serum reacted strongly, resulting in a higher signal than was attained with the affinity purified IgY. Seven other sera, negative for CCPP, were also tested and gave inconsistent results (not shown) making the assessment very difficult, as a suitable negative control was not available.

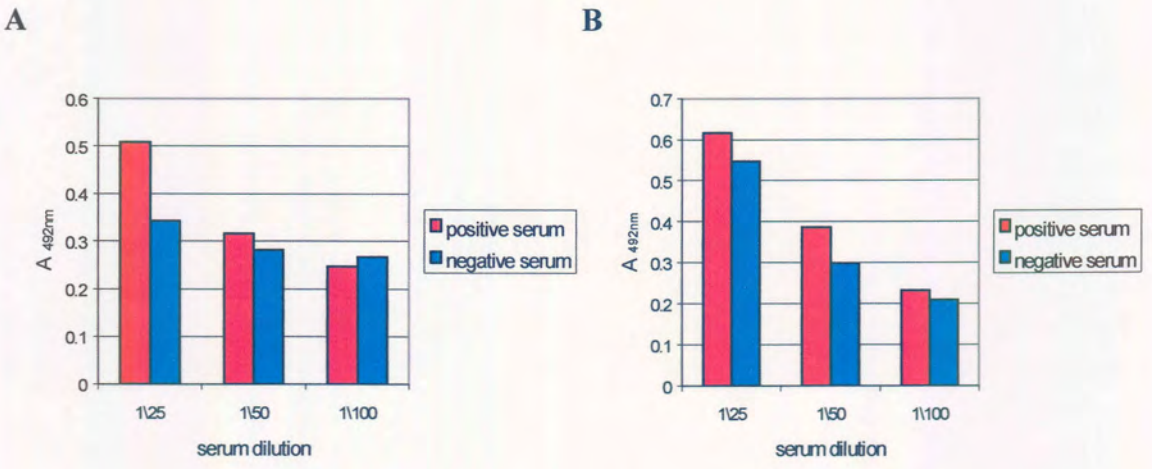


Figure 2.4.19

Evaluation of anti-idiotype IgY by detection of binding to affinity purified IgY by immune goat anti-serum (93.1). A concentration of 10 µg/ml of affinity purified IgY was coated onto microtitre plate wells (A). Pre-immune IgY was coated onto adjacent wells as a control (B).

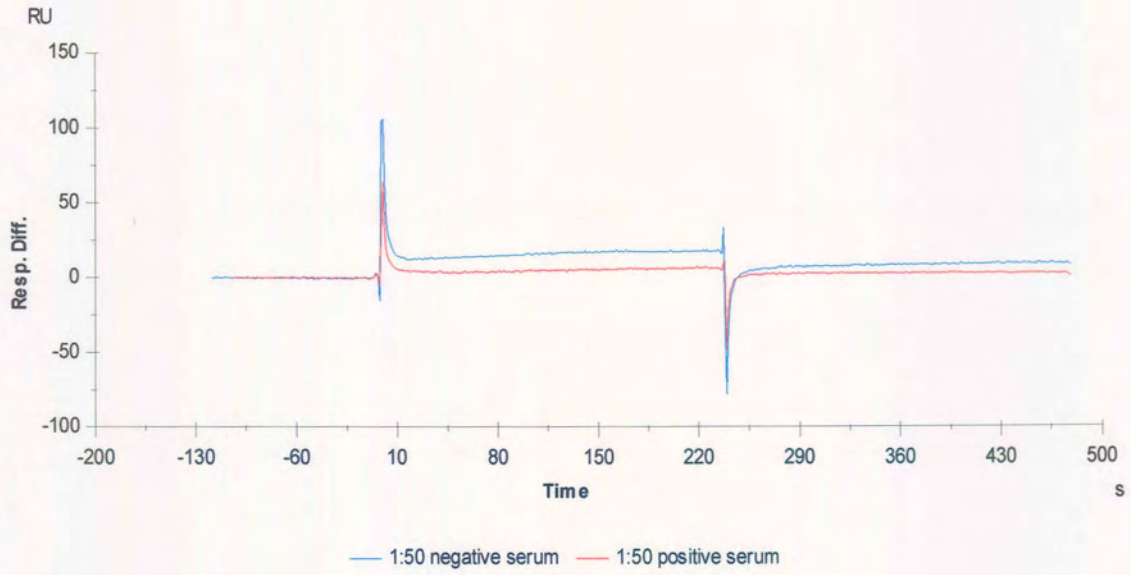


Figure 2.4.20

Overlay plot of the binding curves of Mccp positive (93.1) and negative goat anti-serum to immobilised affinity purified IgY. A volume of 40 µl of serum diluted in HBS buffer was injected at a flow rate of 10 µl/min. The responses shown were reference subtracted with the uncoupled dextran surface of flow cell 1.

Additional to serum 93.1, two other Mccp positive and negative goat antisera were tested in an indirect ELISA with the immunoaffinity purified IgY by Dr F. Thiaucourt (CIRAD-EMVT, France). The outcome was similar as neither positive antisera showed any difference in the intensity of signal when compared with Mccp negative sera.

2.5 Discussion

The use of anti-idiotype antibodies as diagnostic reagents was first reported by Potocnjak *et al.* (1982) for *Plasmodium berghei*, a parasite that causes malaria. The authors used an inhibition test in which radiolabelled anti-idiotype antibodies competed with the antigen from binding to the solid-phase antibodies. A more recent study using anti-idiotype antibodies as serodiagnostic reagents for bluetongue virus (BTV) infections employed anti-idiotype antibodies directly adsorbed to the solid-phase of ELISA plates (Zhou, 1999). The response generated in the mice from both groups immunised with Mab 4.52 appeared to be predominantly against isotypic and allotypic determinants on the Mab. In ELISA, the signal disappeared completely after cross-absorption of the mouse serum with Mab p4.4 (figure 2.4.7) and none of the ELISAs showed any residual antibody activity that could be attributed to interaction with Mab 4.52 idiotopes. Even though the induction of an Ab2 response in syngeneic models is not frequently observed (Ismaili *et al.*, 1995), autologous Ab2 immune responses have been generated by Mabs directed to haptens and self-antigens. In many of the cases the Mabs were administered coupled to KLH (keyhole limpet hemocyanin) to induce an evident Ab2 antibody response (Battacharya-Chatterjee *et al.*, 1987).

The murine Mab 4.52 could be expected to have low immunogenicity in mice. It was perhaps not surprising that the mouse's immune system ignored the antibody. An animal avoids mounting an immune response against 'self' antigen by eliminating B cells or T cells that produce receptors that can bind to host molecules. Tolerance can be induced by the removal of a particular antigen-specific clone of either B cells or T cells, although T cells tolerance is more common. Despite the above, this approach still has the potential to be successful. For instance Mab 4.52 can be coupled to KLH to increase its immunogenicity in mice and by using standard hybridoma technology, it could be possible to generate monoclonal anti-idiotype clones. There are several attractive features of making monoclonal Ab2s: they are homogeneous, maintain their properties with time and can be obtained in unlimited amounts. If these antibodies are the image of the foreign epitope and if the epitope had a protective function, protective immunity against a

particular disease could be induced if the animal was immunised with the Ab2. An alternative to producing monoclonal Ab2s by using hybridomas would be to construct a phage display antibody library. This could be done by amplifying the immunoglobulin variable genes of B cells from the spleen of a mouse immunised with Mab 4.52 and cloning the variable (V) domains into a phage display expression vector. This library could then be panned on immobilised Mab 4.52 to select Ab2s.

The generation of Ab2s in chickens has been less explored (Siskind *et al.*, 1983; Ainsworth and Brown, 1991; Zhang and Ainsworth, 1994), but the approach has yielded promising results. The immunised chickens were found to be efficient producers of antibodies against the $F(ab')_2$ fragments of Mab 4.52, but only a small proportion of the total antibody population was directed against the idiotope. The population of antibodies appeared to mimic the molecular contacts that exist between antigen and antibody as the total IgY preparation was able to inhibit the binding of Mab 4.52 to the Mccp antigen (figure 2.4.8). In order to avoid detecting antibodies binding to the anti-murine allotypic and isotypic determinants, the IgY preparation was treated by passing it through an immunoaffinity column to remove allotypic and isotypic reactivities. Mab 4.52-specific immunoglobulins were, however, not efficiently recognised amongst an overwhelming number of non-anti-idiotype antibodies. This was shown by the experiment in which IgY coated onto the mictrotitre plate surface did not bind sufficient of Mab 4.52 to produce an ELISA signal, but in the opposite orientation, the Mab could capture detectable quantities of anti-idiotype IgY (figure 2.4.10). Since this preparation did appear to contain anti-idiotype IgY, further efforts were made to decrease the proportion of non-idiotype antibodies. The IgY was therefore immunoaffinity purified on Mab 4.52. The rationale was to isolate antibodies specific to Mab 4.52 idiotype determinants and so increase the desired epitope density. Based on immunochemical criteria, the IgY that had been both affinity purified and depleted of mouse allotype and isotype antibodies did behave as a surrogate antigen in ELISA (figure 2.4.15) and in Biacore analysis (figure 2.4.17). This would suggest that the IgY possessed the characteristics of either β- or γ-type Ab2s (Jerne, 1974; Bona and Kohler, 1984). These Ab2s recognise paratope associated idiotopes and are therefore able to inhibit the binding of Mab to the antigen. The β-type

Ab2 represents an internal image, which mimics the three-dimensional structure of the antigen identified by the Mab whereas the γ-type cannot mimic the antigen. Instead, it recognises idiotypic determinants closely associated with the paratope thereby interfering with the binding of the antigen.

Goat antiserum (93.1) raised against Mccp and used as a control in a standardised test for Mccp antibodies (Thiaucourt *et al.*, 1994) was unable to recognise the anti-idiotype IgY. One of the problems inherent when working with goat serum seems to be its innate 'stickiness', which is evident in the results obtained (figure 2.4.19). The ELISA response was largely due to non-specific binding of the anti-serum to the IgY, as immobilised pre-immune IgY resulted in similar if not higher signals. The signals obtained were therefore considered to be background. Similarly, binding analysis of the Mccp positive and negative antiserum in the Biacore also produced identical responses on immobilised affinity purified IgY and on the uncoupled surface of the reference flowcell (figure 2.4.20).

All immunoassays, including the indirect ELISA performed by Dr. F. Thiaucourt, indicated that the goat antisera did not bind specifically to the Ab2s, however, it appeared that the Ab2s were recognised by the Mab 4.52's paratope. It is possible and indeed likely, that the population of anti-idiotype antibodies isolated from the egg yolk contains few or no β-type anti-idiotype antibodies. Inhibition of Mab 4.52 binding to the cell lysate would then have been as a result of the Ab2s binding close to Mab 4.52's paratope with high affinity, so blocking access to the epitope on Mccp. To affirm that the population of antibodies produced by the chicken was not devoid of anti-idiotype β-type IgY, the antibodies would need to be injected into another animal and the antibodies so generated tested for Ab3 activity (anti-anti-idiotype antibodies). In this way, if the antibodies generated are the image of the Mab, it provides conclusive proof that the Ab2s did in fact contain anti-idiotype β-type IgY. Alternatively, to find Ab2s, a phage display antibody library could be constructed using the mRNA from the spleen of the chicken immunised with Mab 4.52. One advantage of phage display recombinant antibodies is that they can be modified by mutagenesis to improve their affinity for the Mab.

Chapter 3

Phage display

Nothing is wasted, nothing is in vain:/
the seas roll over, but the rocks remain.
A. P. Herbert, 1890-1971

Parts of this chapter have been prepared and submitted for publication:
Phage displayed peptides and anti-idiotype antibodies recognised by a monoclonal antibody directed against a diagnostic antigen of *Mycoplasma capricolum* subsp. *capripneumoniae*
Dubravka R. Benguric, Baptiste Dungu, François Thiaucourt and Dion H. du Plessis
Veterinary Microbiology

3.1 Introduction

As discussed in the previous chapter, an anti-idiotype antibody raised against a relevant epitope has the potential to be used as an alternative antigen to whole microorganisms in assays. Phage display technology can also be used to identify and isolate surrogate antigens. Peptides are selected from a library containing millions of phages, each displaying a unique peptide, by binding to a target molecule.

The filamentous phage vector f88.4 was originally derived from fd-tet (Zacher *et al.*, 1980). It has a tetracycline resistance determinant and by adding a synthetic recombinant gene VIII with *HindIII* and *PstI* restriction sites, it allows foreign DNA to be fused to its coding sequence. Transcription of the recombinant gene is controlled by an isopropyl-1-thio-β-D-galactopyranoside-inducible (IPTG) *tac* promoter, whereas the wild-type gene is transcribed constitutively. When both are expressed, the phage particles are covered with a mosaic of wild-type and recombinant pVIII molecules. The foreign peptide on the recombinant pVIII starts just downstream of the presumed signal peptidase cleavage site. Theoretically therefore, it is displayed with a free N-terminus. Its coding sequence can be determined with a primer complementary to the recombinant gene, but not the wild-type one (Zhong *et al.*, 1994). Unlike phagemid vectors, f88.4 has the advantage of not needing a helper phage as well as allowing for better discrimination between weak- and strong-binding peptides, since recombinant pVIII-displayed peptides are not displayed on every pVIII of the virion (Bonnycastle *et al.*, 1996).

Constrained libraries have been generated in which a random peptide sequence is inserted, generally between fixed cysteine residues (Luzzago *et al.*, 1993; Zhong *et al.*, 1994). It is argued that constrained peptides may better approximate the structure recognised in the native protein. In several instances, two libraries displaying either unconstrained random peptides or disulfide-constrained ones have been screened by a monoclonal antibody (Felici *et al.*, 1993; Hoess *et al.*, 1993). Relatively tight binding peptides were selected from only one of the two libraries screened, this indicates that the preference for a given library, in yielding the best-binding peptides, does depends upon the structural framework within which a random peptide is presented.

The XCX_{15} phage display library of Bonnycastle *et al.*, (1996) contains 10^9 different peptide sequences displayed as a fusion with the gene VIII protein of bacteriophages. This library was used to select peptides that bind to Mab 4.52. Their suitability as surrogate epitopes for the determinant, which the Mab recognises, was investigated.

3.2 Materials

3.2.1 Bacterial strain, epitope library and primer

K91 *Escherichia coli* cells was kindly provided by Dr. G. P. Smith (University of Missouri, USA). The XCX_{15} phage display library and the phage display vector f88.4 were gifts from Dr. L. Bonnycastle (Simon Fraser University, Burnaby, BC, Canada). An 18 base pair primer ($^{3'}$ CGA AAA CTG AGA GAA GTC $^{5'}$) specific to the f88.4 vector's (Zhong *et al.*, 1994) synthetic gene VIII, which hybridises 30 base pairs downstream of the multiple cloning site was purchased from Whitehead Scientific.

3.3 Methods

3.3.1 Starved cells

A single colony of K91 *Escherichia coli* was inoculated into 2 ml of LB-broth and incubated overnight at 37°C with vigorous shaking. A volume of 500 µl of the overnight culture was inoculated into 20 ml of LB-broth and grown with slow shaking (70 rpm) at 37°C to an optical density (600 nm) of approximately 0.45. Care was taken in subsequent steps to avoid shearing the fragile F pili. The culture was centrifuged for 10 minutes at 2200 rpm, the supernatant poured off and the cells gently resuspended in 20 ml of 80 mM NaCl. A 125 ml culture flask containing the cells was gently shaken for 45 minutes at 37°C to starve the cells. Thereafter cells were collected by centrifugation for 20 minutes at 2200 rpm and resuspended in 1 ml of cold NAP buffer (appendix). The cells were stored at 4°C and remained viable for 3-4 days.

3.3.2 Affinity selection

In the first round of selection the target molecule was coated onto a Petri dish (Falcon 1007) (in PBS, overnight at 4°C). The next day nonspecific binding sites were blocked for 1 hour at 37°C with TRIS-buffered saline (TBS) containing 2% bovine serum albumin (BSA) (TBS/BSA). After washing the Petri dish three times with TBS containing 0.05% Tween 20, approximately 10^{11} transducing units (TU) of the epitope library was added to the solid-phase for 1 hour at 37°C in 1% BSA/TBS/0.05% Tween 20. Unbound phages were removed by washing the Petri dish five times with

TBS/0.05% Tween 20, five times with TBS/0.5% Tween 20 and once with TBS. Bound phages were eluted in 800 µl of elution buffer (0.1 N HCl, pH adjusted to 2.2 with 100mM glycine, 1 mg/ml BSA) at room temperature for 10 minutes. This eluate was neutralised with 96 µl 1 M Tris/HCl pH 9.1. The Petri dish was washed twice with TBS to restore the pH, after which remaining bound phages were eluted in 1 ml of 0.1 M triethylamine pH 12 for 30 minutes. The eluate was aspirated and neutralised with 500 µl of 1 M Tris/HCl pH 7.4. The neutralised eluates were individually concentrated by filtration in Ultrafuge (MSI) tubes (30 000 MW cutoff) to a volume of 200 µl each. The selected phages were amplified by infecting, for 10 minutes at room temperature, 200 µl of starved K91 cells. One millilitre of LB-broth containing 0.2 µg/ml tetracycline was added to the infected cells and incubated for 1 hour at 37°C with shaking. A volume of 100 µl of each dilution of amplified eluates was spread onto LB agar plates containing 40 µg/ml of tetracycline to quantify the output of the affinity selection. The remaining undiluted eluates were plated onto 24 x 24cm LB agar plates (40 µg/ml tetracycline) and incubated overnight at 37°C. The output of phages was determined by substituting in the formula:

TU = CFU X DF X TOTAL VOLUME / VOLUME PLATED where,

TU = Output

CFU = number of colony forming units

DF = dilution factor

Subsequent rounds of selection were performed in the same way, except that a 50 µl volume of the target molecule was immobilised onto a microtitre plate well (Nunc PolySorp™). The volume of all the reagents used were adjusted accordingly.

3.3.3 Harvest and purification of phages

Bacterial colonies were scraped off the agar surface and the cells pelleted by centrifugation for 10 minutes at 5000 rpm. The pellet was discarded and the supernatant centrifuged for an additional 10 minutes at 8000 rpm. The phages were precipitated by adding 0.15 the supernatant volume, 16% PEG/3.3 M NaCl solution to the supernatant and incubated at 4°C overnight. The PEG precipitated phages were collected by centrifugation for 40 minutes at 8000 rpm, the supernatant was discarded and the phage pellet centrifuged for an additional 10 minutes at 8000 rpm. The pellet

was dissolved in 1 ml of TBS, and centrifuged for 10 minutes at 14 000 rpm. The phages were precipitated for a second time, again by adding 0.15 the supernatant volume 16% PEG/3.3 M NaCl solution to the supernatant and incubating the solution at 4°C for more than 1 hour. The phages were collected by centrifugation at 14000 rpm and the pellet resuspended in 100 µl of TBS.

The concentration (C) of physical particles (mg protein/ml) was determined by scanning a 1/100 dilution of phages from 240 nm to 320 nm using a HEλIOS UNICAM UV-Vis spectrometer. An absorbance of 30 at 269 nm for undiluted phage corresponds to a DNA content of 1 mg/ml. Since there is approximately six times more viral protein than viral DNA by mass (Smith, 1992), the concentration was calculated by using the following formula: $C = 1/30 \text{ X DF X } 6 \text{ X } A_{269\ nm}$

3.3.4 Colony Lift

Bacterial colonies were randomly picked and transferred onto LB agar plates (40 µg/ml tetracycline). A nitrocellulose membrane (Hybond™-C, Amersham) was placed onto the colonies and removed after 5 minutes, and rinsed in PBS. The membrane was immersed in blocking solution (PBS containing 2% milk powder) to block nonspecific binding sites for approximately 30 minutes at room temperature. Thereafter, 4 ml of primary antibody, diluted in PBS containing 1% milk powder solution, was added at a concentration of 5 µg/ml and incubation was for 1 hour at room temperature. Next, the membrane was washed three times for 5 minutes with PBS containing 0.05% Tween 20 and probed with appropriate HRP-conjugated immunoglobulins for 1 hour. The membrane was washed as before and equal volumes of SuperSignal® Chemiluminescent substrate (PIERCE) reagents were mixed and added to the membrane for 5 minutes. The image was recorded using a LumiImager (Roche).

3.3.5 Sequencing templates

Positive clones identified by the colony lift were picked and inoculated in 15 ml of LB-broth containing 40 µg/ml of tetracycline, and incubated overnight at 37°C with vigorous shaking. The phages were precipitated with PEG/NaCl solution as described in 3.3.3. A volume of 60 µl of the PEG precipitated phages were diluted with

deionised water to a final volume of 100 µl. An equal volume of phenol/chloroform/isopropanol (50:49:1) was added and the mixture vortexed for 15-20 seconds and incubated at room temperature for 15 minutes. The mixture was vortexed again and approximately 100 µl of phase lock gel (5Prime→3Prime Inc.) was added to each tube and centrifuged for 3 minutes at 14000rpm. The top aqueous layer containing the ssDNA was transferred to a new tube and precipitated by adding 10 µl of 3 M NaOAc and 250 µl of ice-cold 100% ethanol. After 30 minutes at -70°C, the ssDNA was collected by centrifugation for 10 minutes at 14000 rpm. The supernatant was aspirated and 1 ml of 70% ethanol added to wash the pellet. The ssDNA was air-dried, resuspended in 10 µl of TE buffer and stored at -20°C.

3.3.6 Agarose gel electrophoresis

The relative concentration of ssDNA was measured by agarose gel electrophoresis. A 0.7% agarose gel was prepared by dissolving 0.21 g of agarose in 30 ml of 1X TAE buffer. Ethidium bromide was added and the molten agarose was poured into a gel tray and allowed to set with a comb in place. Once the gel had set, the comb was removed and the tray placed in a standard gel electrophoresis apparatus containing 1X TAE as the running buffer. A volume of 3 µl of each ssDNA sample was individually mixed with 4 µl of loading buffer and electrophoresed at 100 V, until the blue dye had migrated halfway through the gel. The ssDNA was viewed on an UV transilluminator.

3.3.7 Manual sequencing

i) Sequencing reactions

Primer annealing reactions were prepared by adding 7 µl of sequencing template, 1.4 µl of deionised water and 2.4 µl of reaction buffer (Amersham) to 1 µl of primer (1 pmol/µl). The reactions were incubated at 70°C for 10 minutes, followed by 10 minutes at 37°C and 10 minutes at room temperature. The extension reaction (for 18 samples) was prepared by adding 9 µl of 5X labeling mix (Amersham), 6 µl of 0.1 M dithiothreitol, 12 µl of ^{35}S-dATP (10 mCi/ml, Amersham), 18 µl of Mn^{2+} buffer and 4.5 µl of T7 sequenase polymerase (Amersham) to deionised water to a final volume of 90 µl.

Five microlitres of the extension reaction was added to each sample's annealed reaction and incubated at room temperature for 5 minutes. A volume of 2.5 µl of each termination mix (ddG; ddT; ddC; ddA, Amersham) was pipetted into microtitre plate wells (Costar, serocluster™ "U" vinyl plate) for each sample. A volume of 3.5 µl of the annealed and extension reaction mix was added to each termination mix and incubated at 37°C for 10 minutes. The reactions were stopped by adding 4 µl of stop solution (Amersham) to each reaction and stored at -20°C.

ii) Sequencing gels

Standard manual sequencing methodologies were used throughout this study (Sambrook *et al.*, 1989). Sequencing gels (380 x 305 x 0.4 mm) were run in an OWL sequencing gel apparatus. The notched glass plate was siliconised with approximately 5 ml of windshield rain dispersant. The plates were wiped with 99% ethanol and buffed with a paper towel then clamped together, 0.4 mm spacers were placed between but along the sides of the plates and a piece of filter paper between but along the bottom of the plates. The 6% sequencing gel solution was made up to 100 ml with deionised water and contained 42 g urea, 10 ml of 10X TBE and 15 ml of 38% acrylamide/2% bis-acrylamide solution. Two hundred microlitres of 10% freshly dissolved ammonium persulphate was added to the gel, followed by 80 µl of TEMED. The gel was immediately poured between the plates. The non-serrated edge of the 0.4 mm comb was inserted approximately 1 mm into the gel. Once the gel had polymerised it was assembled onto a sequencing gel electrophoresis apparatus. The serrated edge of the comb was pushed approximately 3 mm into the gel to create 6 mm wide wells. The gel was pre-electrophoresed (70 W) until the surface temperature of the plates reached approximately 50°C.

Before each sample was loaded onto the gel the sequencing reactions were incubated for 5 minutes at 90°C to denature the DNA and the wells were rinsed with running buffer (0.5X TBE) to remove residual urea. A volume of 1-1.5 µl of sample was loaded per well and electrophoresed until the xylene cyanol FF (blue-green) band had migrated three quarters of the gel length. After electrophoresis, the plates were prided apart and the gel absorbed to filter paper by pressing the filter paper firmly onto the gel, which remained on the non-siliconised plate. The gel was then lifted off the glass plate by lifting the filter paper and vacuum-dried for approximately 2 hours at 80°C.

Hyperfilm™ (Amersham) was exposed to the dried gel in a film cassette overnight at room temperature. The film was developed under ambient light by immersing it in 10% developer (Ilford), rinsing with deionised water, immersing it in 25% fixer (Ilford) and finally rinsing again with deionised water.

3.3.8 Indirect ELISA

Wells of a microtitre plate (Corning) were coated overnight at 4°C with 10 µg/ml of antigen. After coating, the wells were incubated at 37°C with 300 µl of PBS containing 5% milk powder as blocking solution, before washing five times with PBS containing 0.1% Tween 20. Primary antibody was diluted in PBS containing 5% milk powder and 0.1% Tween 20 to 10 µg/ml. A volume of 50 µl was added to the solid-phase for 1 hour at 37°C after which the wells were washed as before and 50 µl volumes of appropriate HRP-conjugated immunoglobulins were added for 1 hour at 37°C. Following washing, antibody binding was detected by adding a peroxidase substrate, consisting of 10 mg of o-phenylenediamine dihydrochloride in 10 ml of 0.1 M citrate buffer pH 4.5 and 5 µl of 30% hydrogen peroxide to each well. The reaction was stopped after a minimum period of 10 minutes with the addition of 50 µl of 2 N sulphuric acid and the absorbance (492 nm) was measured using a BDSL Immunoskan MS ELISA reader.

3.3.9 Surface plasmon resonance

An F1 Pioneer chip (Biacore AB, Sweden) was docked and primed in the BIACORE® X system. A single mode of detection was chosen over flow path 2 at a flow rate of 5 µl/min. Phages to be immobilised via the amine-coupling procedure were diluted to 200 µg/ml in acetate buffer pH 4. All procedures were performed at 25°C. The chip surface was activated by loading the sample loop and injecting 35 µl of a mixture of NHS and EDC, followed by 40 µl of phage. A volume of 35 µl of 1 M ethanolamine hydrochloride pH 8.5 was injected to deactivate excess reactive groups once the coupling procedure was complete. This was followed by an additional pulse of 5 µl of deactivation solution to condition the surface before performing a run. Ligand to be used as a control was immobilised in flow cell 1 using the same procedure. Samples were diluted in HBS buffer (Biacore AB, Sweden) and injected

over the chip surface at a flow rate of 10 µl/min. The surface was regenerated after each injection with 10 µl of 100 mM phosphoric acid.

3.3.10 Inhibition ELISA

The ELISA was performed essentially as described in 3.3.8, except that various concentrations of equal volume of two antibody containing solutions were individually mixed at room temperature for 1 hour. The mixture was added to antigen coated wells for 1 hour at 37°C. Thereafter, HRP-conjugated immunoglobulins were added for 1 hour at 37°C to detect the binding. Percentage inhibition (P.I) was calculated using the formula:

$P.I = A_0 - A_{SAMPLE} / A_0 - A_{100} X 100$

A_{100} = 100% inhibition

A_0 = 0% inhibition

3.3.11 Blocking ELISA

The blocking ELISA was performed as described in 3.3.8 with the exception that primary antibody was added to the solid-phase immobilised with antigen for 1 hour at 37°C. This was followed by adding a secondary antibody for 1 hour at 37°C after which binding was detected by HRP-conjugated antibodies for 1 hour at 37°C.

3.4 Results

To search for peptide mimics of the epitope recognised by the Mccp-specific Mab 4.52 (Thiaucourt *et al.*, 1994), a collection of 17- mer pVIII fusion peptides displayed on the surface of a library of filamentous phages (Bonnycastle *et al.*, 1996) was subjected to immunoaffinity selection by panning on the immobilised Mab 4.52. Two pannings were performed, each consisting of four rounds of selection.

In the first panning experiment, protein A was immobilised onto a Petri dish (Falcon) to which Mab 4.52 was then added. By binding to the Fc, this theoretically allowed the antibody's variable domains to be better exposed to the phage library. In round 2, protein G was immobilised followed by the Mab. In this way consecutive enrichment for fusion phages with an affinity for protein A was circumvented. Mab 4.52 was directly immobilised onto microtitre plate wells for rounds 3 and 4. The concentration of the Mab was decreased to a tenth of the concentration used in round 3, to increase the stringency of selection and so enrich for higher affinity fusion phages. Table 1 shows the percentage output after each round of selection. There was a gradual increase in the percentage output of binders from round 1 to round 3 followed by a thousand fold increase in enrichment for binders in round 4. After rounds 3 and 4 of selection, 50 bacterial colonies were randomly picked and spotted onto LB agar plates. The resulting colonies and their secreted phages were then transferred to nylon membranes and probed with Mab 4.52 to locate phage clones that displayed antigenic peptides. Table 2 shows the number of positive colonies picked after each round.

A second panning was performed in which Mab 4.52 was directly immobilised onto the solid phase through all four rounds of selection. The percentage output for each round was lower than that achieved in the first panning, but remained higher than 1 X 10^{-5} (Smith, 1992), theoretically indicating specific selection of antigenic peptides for Mab 4.52 (Table 3). Bound phages were sequentially eluted in all rounds with low and high pH buffers. There was no significant difference in the number of positive colonies picked from either elution condition in both panning experiments (Table 2). The DNA of these positive phages was in turn sequenced to identify the displayed peptides. Sequence analysis showed that only two specific sequences (from the

millions of possibilities in the library) were selected (Table 4). Eight clones from round 3 and 21 clones from round 4 of panning 1 were sequenced. All 29 clones revealed the same nucleotide sequence coding for what was designated as peptide X1. From panning 2, 10 clones were sequenced from round 3 of which only one clone had the same nucleotide sequence as peptide X1 from panning 1 (Table 4).

Table 1

Recovery of phages after each round in panning 1.

Round	Antibody concentration	Volume	INPUT (TU)*	OUTPUT (TU)* pH 2.2	OUTPUT (TU)* pH 12	% OUTPUT
1	Mab 4.52 10 µg/ml**	4000 µl	2×10^{12}	1.6×10^{5}	4.5×10^{2}	7.8×10^{-6}
2	Mab 4.52 20 µg/ml***	200 µl	1.1×10^{11}	2.3×10^{4}	2.6×10^{3}	2.3×10^{-5}
	Mab 4.52 20 µg/ml	200 µl		5.1×10^{4}	1.6×10^{4}	6.1×10^{-5}
3	Mab 4.52 10 µg/ml	200 µl	2.9×10^{12}	2.3×10^{6}	2.6×10^{4}	8.0×10^{-5}
4	Mab 4.52 1 µg/ml	200 µl	7×10^{9}	1.6×10^{7}	1.5×10^{4}	3×10^{-2}

***TU = transducing units**
**** Protein A (10 µg/ml) was directly adsorbed to surface followed by the addition of Mab**
***** Protein G (10 µg/ml) was directly adsorbed to surface followed by the addition of Mab**

Table 2

Recovery of phages after each round in panning 2.

Round	Antibody concentration	Volume	INPUT (TU)*	OUTPUT (TU)* pH 2.2	OUTPUT (TU)* pH 12	% OUTPUT
1	Mab 4.52 10 µg/ml	4000 µl	2×10^{12}	3.9×10^{5}	2.2×10^{4}	2.1×10^{-5}
2	Mab 4.52 10 µg/ml	4000 µl	4.1×10^{10}	5×10^{4}	7.7×10^{3}	1.4×10^{-4}
3	Mab 4.52 10 µg/ml	50 µl	1.1×10^{12}	1.2×10^{7}	2.0×10^{6}	1.3×10^{-3}
4	Mab 4.52 1 µg/ml	50 µl	2×10^{11}	1.8×10^{6}	1.7×10^{5}	9.7×10^{-4}

*** TU = transducing units**
**** Where not specified the Mab was directly adsorbed to the surface**

Table 3

Number of positive colonies by colony lift for panning 1 and 2.

Panning 1			Panning 2		
Round	Number of positive colonies		Round	Number of positive colonies	
	pH 2.2	pH 12		pH 2.2	pH 12
3	4/25	4/25	3	10/20	4/20
4	20/25	21/25	4	16/20	18/20

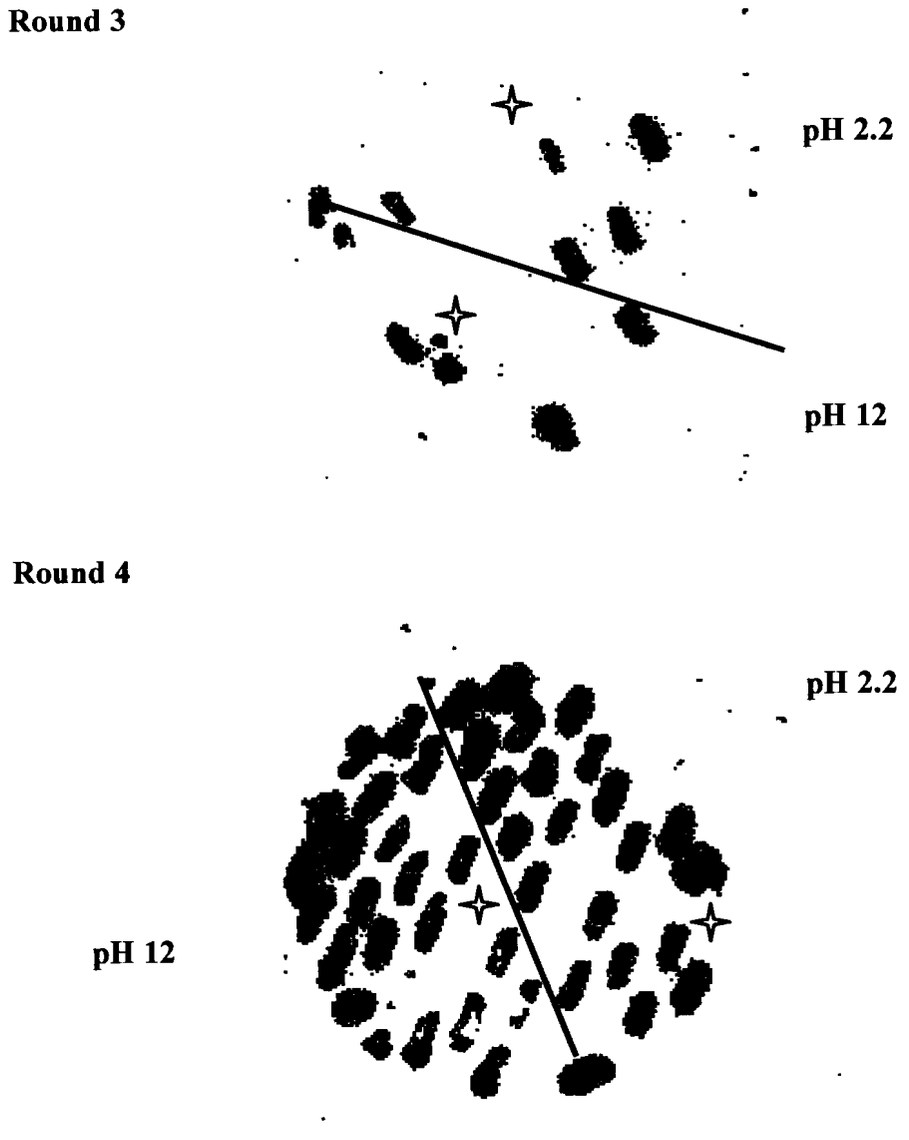


Figure 3.4.1

Colony lift of selected colonies secreting fusion phages from panning 1, rounds 3 and 4. Selected colonies from both elution conditions were spotted onto LB agar plates containing 40 μg/ml of tetracycline. Mab 4.52 (5 μg/ml) binding to fusion phages was detected using rabbit anti-mouse HRP-conjugated antibodies (1:2000, Dako). The yellow stars indicate the position of colonies secreting non-fusion phages (f88.4).

Table 4

Amino acid sequence and frequency of selected fusion peptides displayed by phage clones isolated from the random XCX_{15} library by Mab 4.52.

Type	Amino acid sequence	Panning 1		Panning 2	
		Round 3	Round 4	Round 3	Round 4
X1	SCESPSWIVQYWLRCWV	8	21	1	15
X2	LCKGPDALCLSNLTPML	0	0	9	0

Peptide X2 was not identified in any of the 15 clones sequenced in round 4. Both the selected fusion peptides had a cysteine residue in addition to the one fixed in amino acid position 2, and therefore had the potential to form a loop stabilised by a disulphide linkage. Identical or similar amino acid residues were located at amino acid positions 5 (P), 8, (I/L) and 13 (L) (figure 3.4.2).

```
SCESPSWIVQYWLRCWV
  |   |   |     |
LCKGPDALCLSNLTPML
```

Figure 3.4.2
Alignment of the two fusion peptides identified by panning the random XCX_{15} library with Mab 4.52. Vertical lines indicate amino acid identity or near-identity. The cysteine residues are indicated in blue.

Sequence comparison with the genome sequence database of *Mycoplasma mycoides* subsp. *mycoides* SC strain PG1 by Anja Persson (National Veterinary Institute, Uppsala, Sweden), showed that only peptide X1, the predominant sequence, had significant alignments with the amino acid sequences in the database (figure 3.4.3). Seventy-four percent of the residues of query 1 were similar to peptide X1's residues whereas 64% of the residues of the second match were identical with the fusion peptide.

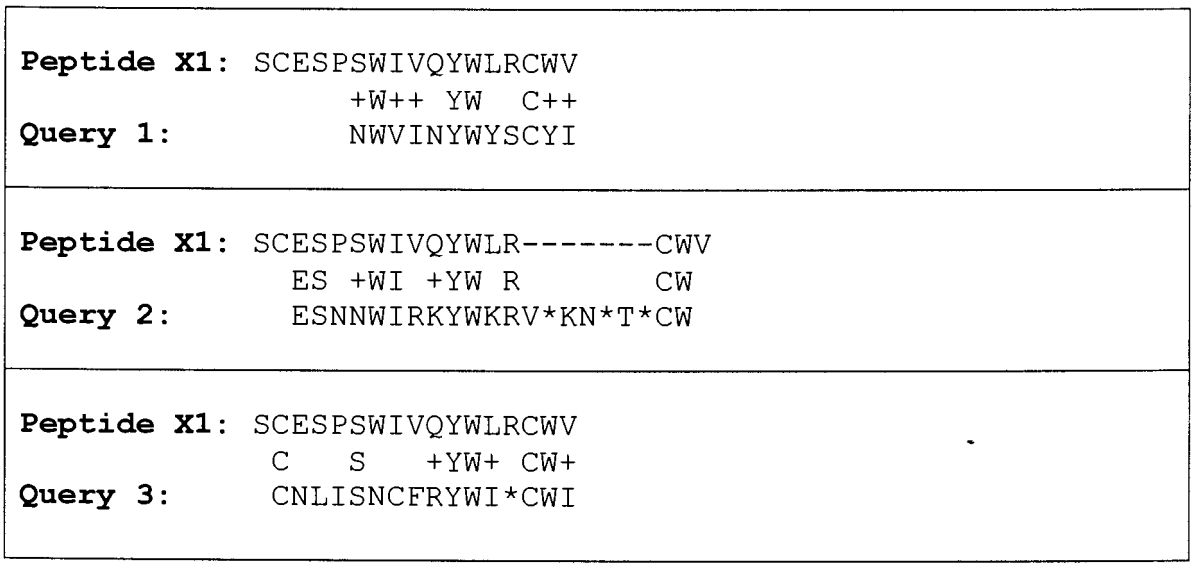


Figure 3.4.3

Amino acid sequence alignments of peptide X1 with *Mycoplasma mycoides* subsp. *mycoides* SC strain PG1 by the Gapped BLAST and PSI-BLAST protein database search program. Query 1 had 4/12 identities and 9/12 positives, query 2, 9/14 identities and 11/14 positives while query 3 gave a match of 6/16 identities and 9/16 positives. Positive matches indicate amino acid residues with similar features or properties.

The antigenicity of the selected fusion peptides was confirmed by indirect ELISA and surface plasmon resonance. In ELISA, peptide X1 produced a significantly lower signal than did peptide X2 (figure 3.4.4). Neither fusion phage reacted with Mab p4.4, an IgG_{2A} bluetongue virus-specific antibody.

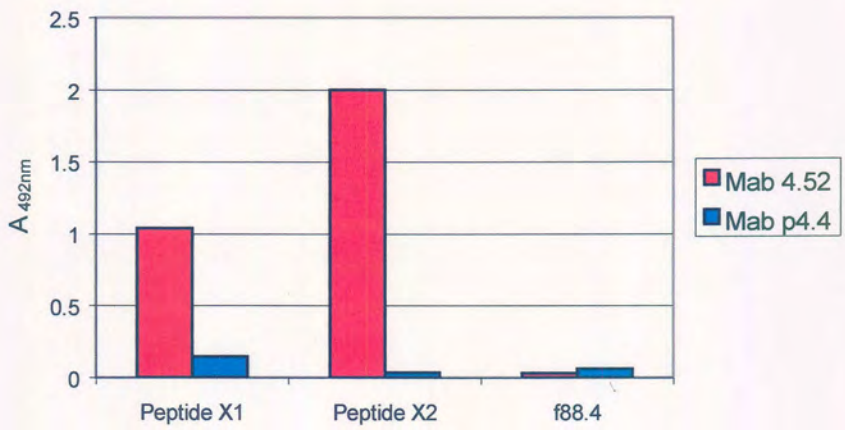


Figure 3.4.4

Antigenic reactivity of fusion peptides X1 and X2 in indirect ELISA. The fusion phages were adsorbed to microtitre plate wells and allowed to react with Mab 4.52. Mab p4.4 was used as a control and bacteriophage f88.4 is the empty display vector. The ELISA results represent the average of duplicate determinations.

The apparent lower affinity seen in ELISA of X1 for Mab 4.52 was confirmed by Biacore analysis in which phage preparations displaying each of the peptides were injected over a sensor chip onto which Mab 4.52 had been covalently attached (figure 3.4.4). Both the apparent rate of association and the maximum response attained were lower in the case of peptide X1. Peptide X2 generated a maximum response of approximately 213 RU whereas peptide X1's response was 70 RU lower.

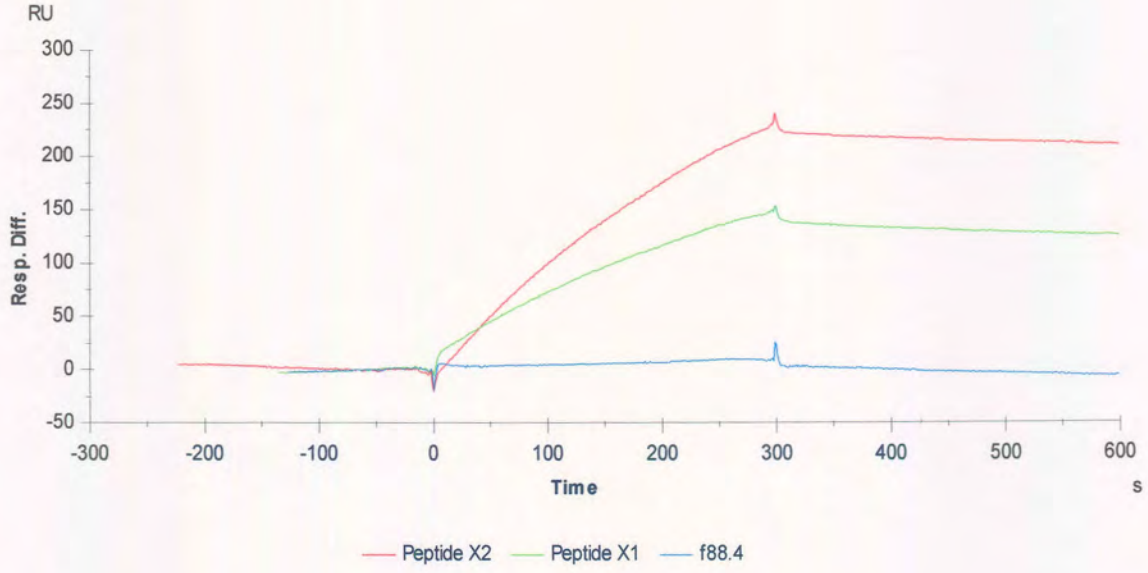
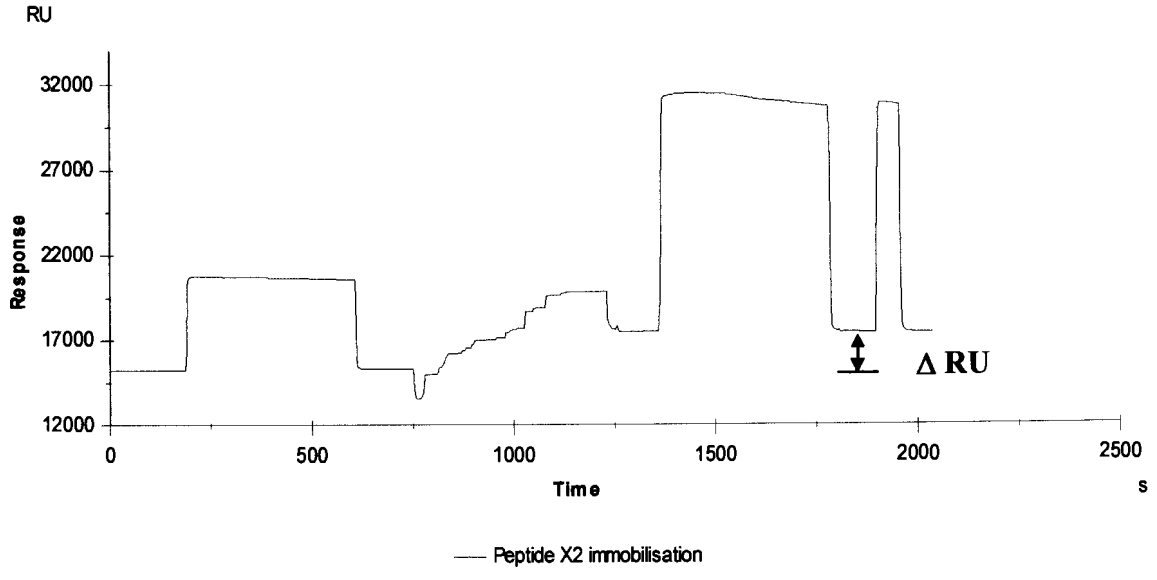


Figure 3.4.5

Overlay plot showing binding of phages displaying fusion peptides X1 (10^{13} virions/ml) and X2 (10^{13} virions/ml) to immobilised Mab 4.52. Fifty microlitre volumes were injected at a flow rate of 10 µl/min. At the end of injection (300 sec) dissociation of the phages commenced. After each injection bound phages were removed with a pulse of 0.1 M phosphoric acid. Bacteriophage f88.4 was used as a control (10^{13} virions/ml). The responses shown are reference subtracted with flow cell 1 immobilised with Mab 1F1.

In a further experiment aimed at confirming binding of Mab 4.52 to X2, phages displaying this peptide were immobilised onto a F1 sensor chip generating an immobilisation level of approximately 2150 RU. Bacteriophage f88.4 was immobilised on an adjacent flow cell yielding an immobilisation level of approximately 500 RU (figure 3.4.6). Mab 4.52 was injected over the immobilised phages, the resulting sensorgram is shown in figure 3.4.7, from which it can be seen that the antibody dissociated relatively rapidly from the phage.

A



B

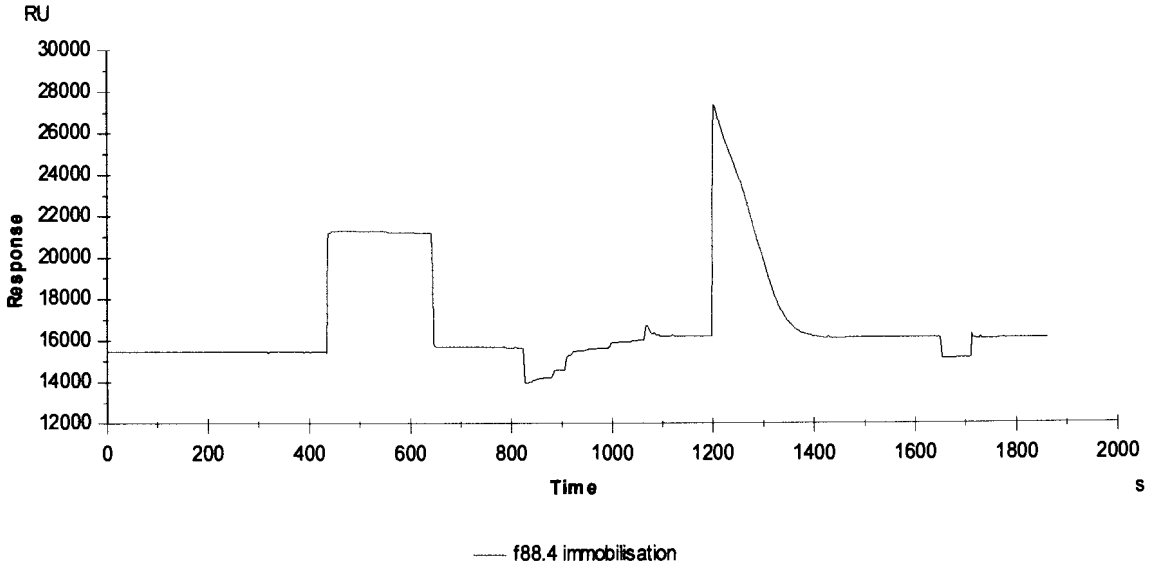


Figure 3.4.6

Sensorgrams showing immobilisation of fusion phage X2 (A) and bacteriophage f88.4, the "empty" display vector (B). ΔRU represents the degree of immobilisation achieved with fusion phage X2, ΔRU for phage f88.4 is 500 RU. The phages were immobilised by injecting 200 μg/ml of each phage diluted in 0.1 M acetate buffer pH 4 at a flow rate of 5 μl/min to the activated surface of a F1 sensor chip.

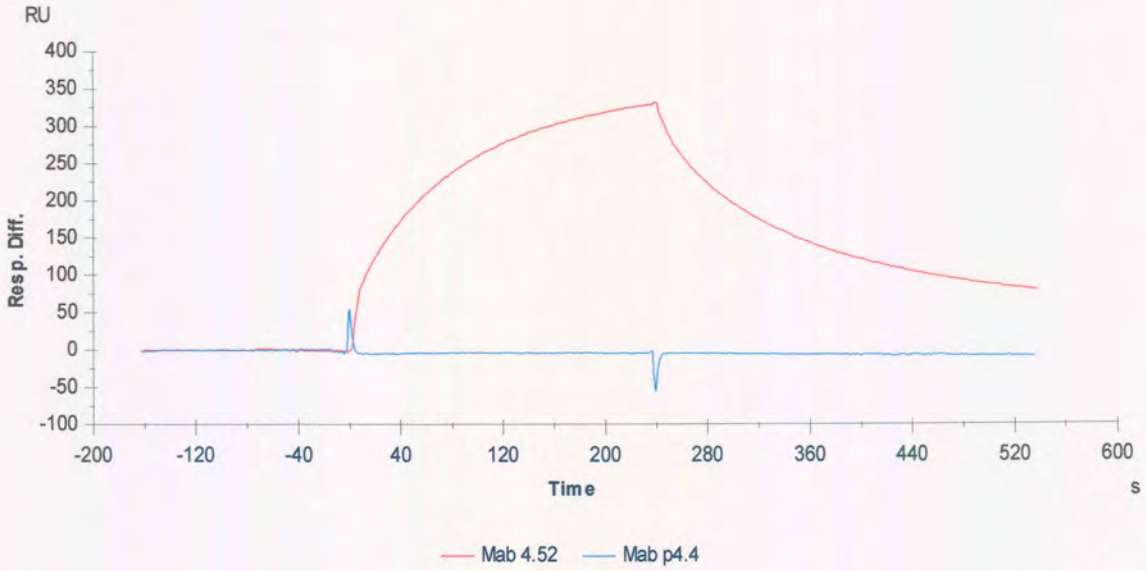


Figure 3.4.7

Overlay plot of two binding curves illustrating the specificity of the selected fusion phage X2. The fusion phage was immobilised to flow cell 2 and the empty display vector to flow cell 1. Both Mabs were diluted to 50 μg/ml in HBS buffer and injected over the surface at a flow rate of 10 μl/min. The responses shown are reference subtracted.

No attempt was made to obtain quantitative data for this interaction, but the dissociation kinetics can be contrasted with the slow release of phages from the immobilised Mab 4.52 (figure 3.4.5). In addition, Mab 4.52 did not recognise the f88.4 phage in ELISA or when the antibody was immobilised on the chip.

An attempt was made to inhibit the interaction of the anti-Mccp Mab 4.52 with its authentic epitope to provide additional confirmation that the selected peptides were in fact being specifically recognised. Mccp cell lysate (Thiaucourt *et al.*, 1994) was adsorbed to the surface of microtitre plate wells and Mab 4.52 was cross-absorbed with the fusion phages before being added. Both antigenic peptide sequences inhibited binding of the antibody to the lysate while non-fusion vector phages did not (figure 3.4.8).

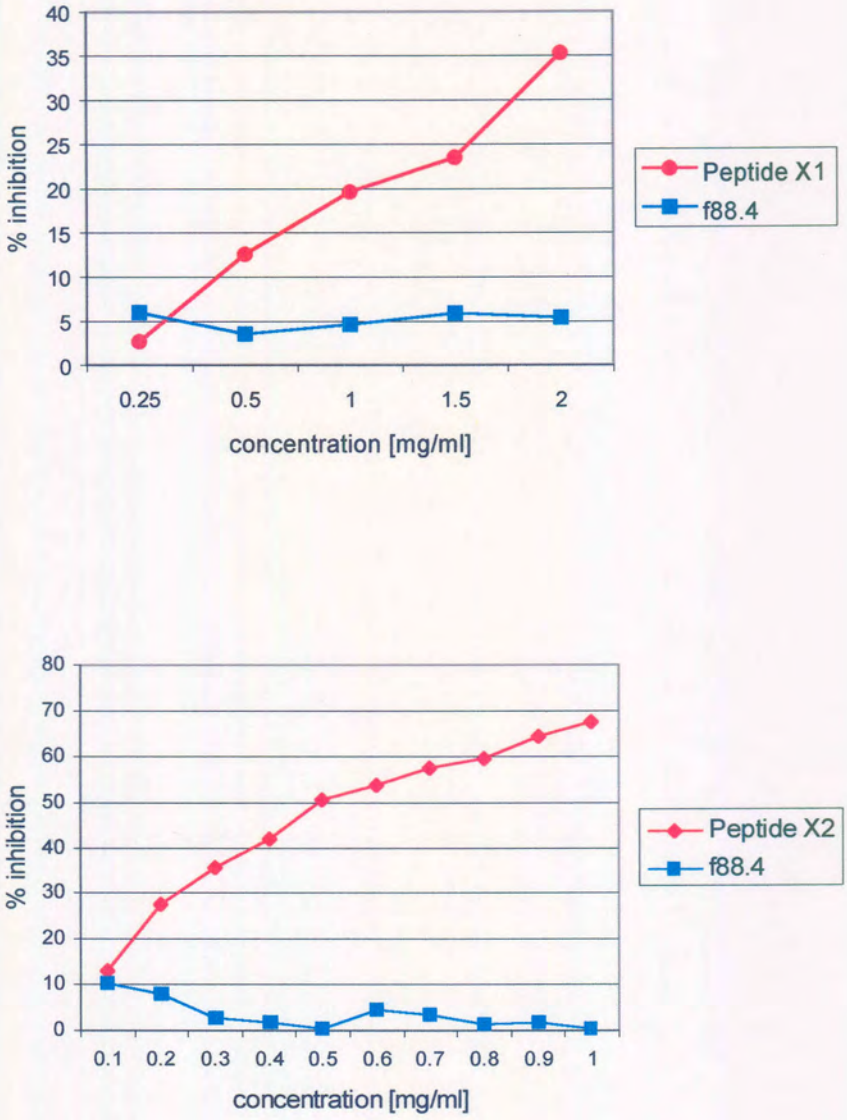


Figure 3.4.8

Competition ELISA in which fusion phages displaying peptide X1 and X2 were allowed to inhibit the binding of Mab 4.52 (1 μg/ml) to Mccp lysate (1:2000) adsorbed to microtitre plate wells. Bacteriophage f88.4 was used as a control. HRP-conjugated rabbit anti-mouse antibodies (1:2000, Dako) detected binding.

In accordance with ELISA and Biacore binding data, peptide X2 was the more efficient inhibitor. Fifty percent inhibition of the ELISA signal was achieved at a phage concentration of 500 µg/ml of X2 as opposed to peptide X1 that was unable to achieve 50% inhibition, even at a concentration as high as 2.0 mg/ml (figure 3.4.8).

Since it was envisaged that they might be useful in diagnosis of the disease, the fusion phages were tested for their ability to bind antibodies in an immune goat serum raised against inactivated Mccp (serum 93.1). Neither was found to be suitable as an ELISA antigen. This was the case in indirect ELISA even at a serum dilution of as high as 1/25 (figure 3.4.10). The strongly binding peptide X2 was also tested in an inhibition format where the goat antiserum was allowed time to block binding of the Mab to immobilised phages (figure 3.4.9). Serum 93.1 was unable to block the Mab binding to the lysate. Moreover, in the Biacore, no specific binding of the immune goat serum could be demonstrated (figure 3.4.11). Additional to serum 93.1, two other Mccp positive and negative goat antisera were tested in an indirect ELISA with the two fusion phages by Dr. F. Thiaucourt (CIRAD-EMVT, France). The results were similar in that neither positive antiserum showed any difference in the intensity of signal achieved compared with that generated by the Mccp negative sera.

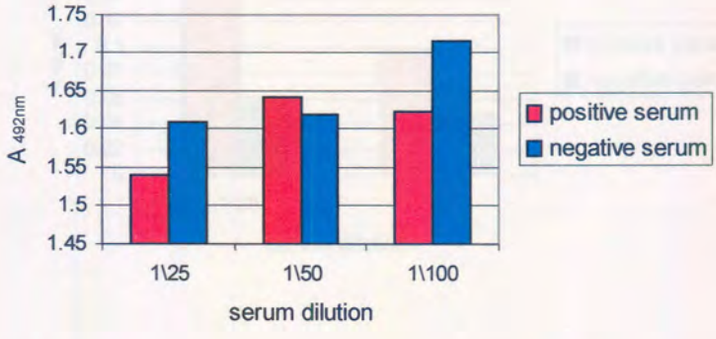
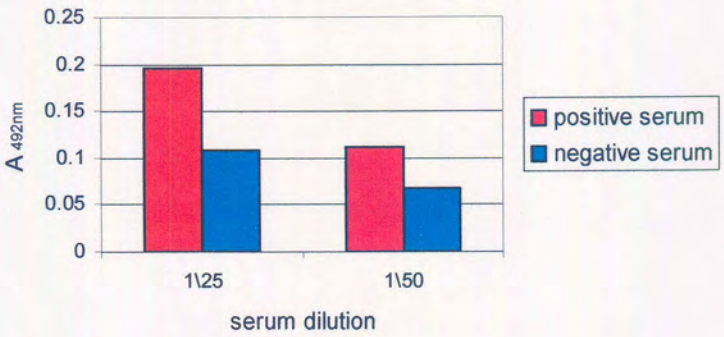


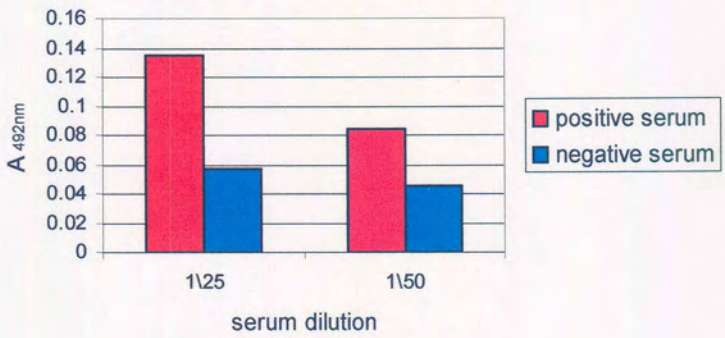
Figure 3.4.9

Testing the suitability of peptide X2 as a surrogate antigen in a blocking ELISA. Fusion phage X2 (10 µg/ml) was coated onto microtitre plate wells in 7 M urea. Goat sera were allowed to react with the peptide after which, Mab 4.52 (10 µg/ml) was added and binding detected with rabbit anti-mouse HRP-conjugated antibodies (1:2000, Dako).

A



B



C

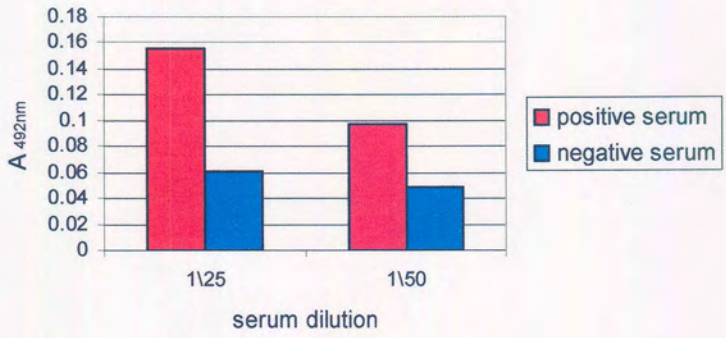
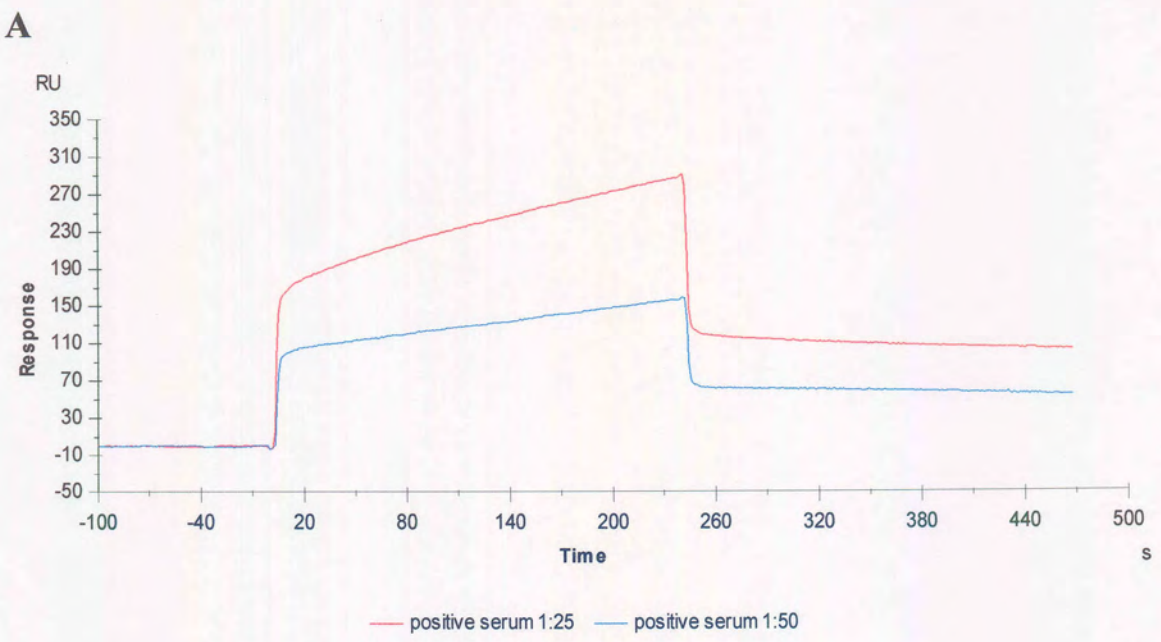


Figure 3.4.10

Indirect ELISA detecting binding of antibodies in goat sera to immobilised phages. Fusion phages X1 (B) and X2 (A) and non-fusion phage f88.4 (C) were coated onto microtitre plate wells (10 μg/ml). Goat sera were cross-absorbed with 500 μg/ml of phage f88.4 and allowed to react with the phage coated wells. Binding was detected with rabbit anti-goat HRP-conjugated antibodies (1:4000, Zymed). The non-fusion phage was used as a negative control.



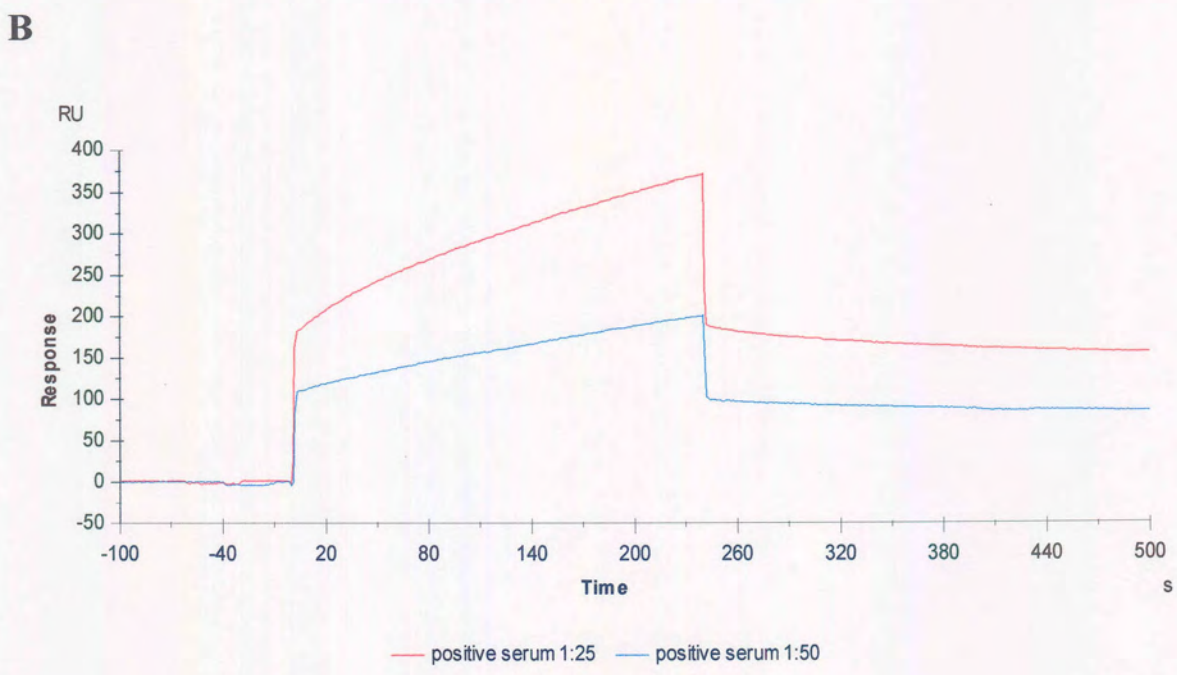


Figure 3.4.11

Overlay plots of binding curves of Mccp positive goat antiserum diluted (1:25 and 1:50) in HBS buffer and injected at a flow rate of 10 µl/min over immobilised fusion phage X2 (A) and non-fusion phage f88.4 (B).

Both the anti-idiotype IgY and the selected fusion peptides recognise Mab 4.52. Biacore analysis shows that the Mab dissociated relatively rapidly from immobilised fusion phages (figure 3.4.7). This could explain the inhibition seen in ELISA (figure 3.4.12) where the fusion phage X2 was coated onto wells and the IgY and Mab were added.

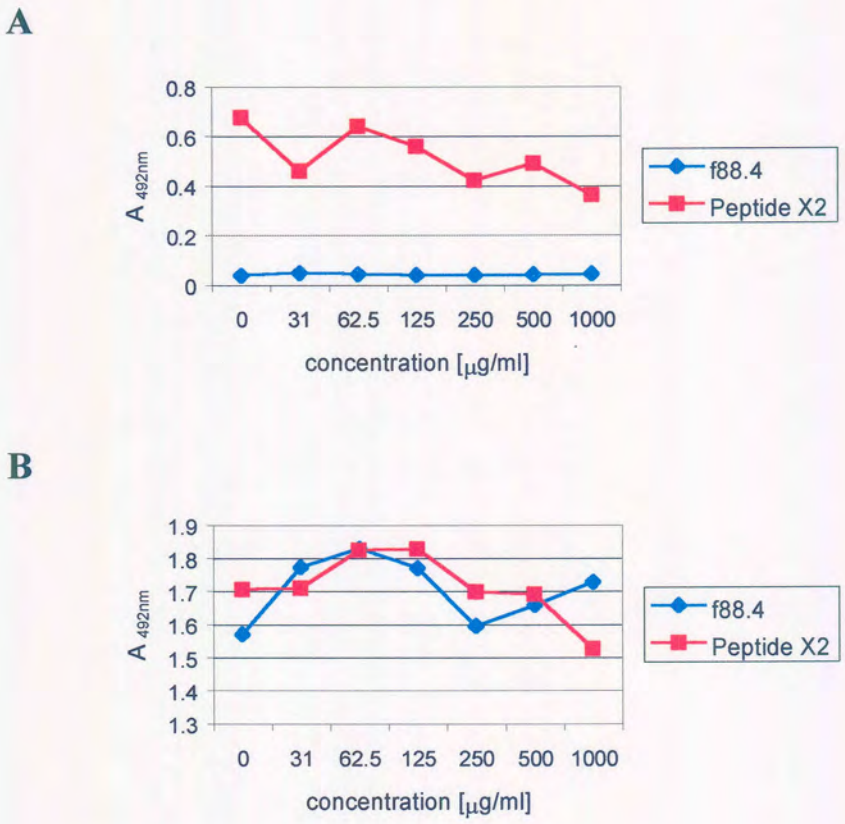


Figure 3.4.12

Competition ELISA showing the reactivity of peptide X2 (A) and anti-idiotype IgY (B) as antigens. In ELISA A, Mab 4.52 (5 µg/ml) was mixed with various concentrations of depleted IgY, in ELISA B, Mab 4.52 (300 ng/ml) was mixed with various concentrations of peptide X2. Bacteriophage f88.4 was used as a control.

Despite this, inhibition was also observed when the IgY is immobilised and the phage and Mab were added. One must, however, take into account that thousands of peptides are presented on a single phage whereas only two binding sites are available on the IgY. It therefore appears that the IgY is a more effective inhibitor than the

fusion peptides. This could be attributed to the slow dissociation rate of the Mab from immobilised IgY (figure 2.4.16) or the conformation of the epitope is better presented on the IgY than by the peptides. It is also possible that the IgY and phage peptides do not recognise the same determinant on the Mab, but a similar one or overlapping regions.

3.5 Discussion

Libraries of random peptides displayed on the surface of bacteriophage have been employed to isolate peptide ligands that bind specifically to a wide range of target ligates and to map epitopes of monoclonal antibodies (Burton, 1995). Using this approach, peptides that bind to Mab 4.52 were isolated from a vast library of random sequences.

The XCX_{15} filamentous phage display library theoretically contains approximately 10^9 different peptide sequences displayed on the gene VIII protein which presents numerous copies of the peptide to the target ligate. From this vast repertoire, only two different peptides were isolated after panning with Mab 4.52. The fact that only these two binding sequences were found is probably as a result of the fact that DNA obtained exclusively from phage clones that had first been shown to bind to Mab 4.52 in colony immunoscreening was sequenced. Biased growth or differing efficiencies of infection by the different fusion phages during replication may have further reduced the final prevalence of other sequences. In addition to being recognised in panning, both fusion peptides were efficient antigens in ELISA. Their binding to Mab 4.52 could also be demonstrated by surface plasmon resonance. In an indirect ELISA with the phages passively adsorbed to the surface of the microtitre plate wells, both peptides bound the *Mycoplasma capricolum* subsp. *capripneumoniae*-specific Mab (figure 3.4.4). Although peptide X2 was not found after the fourth round of selection, perhaps due to biological selection (slower growth), it produced the higher signal in ELISA and gave a correspondingly higher response in the Biacore. The amino acid sequences of the two selected peptides did not show significant similarity. Both sequences, however, did contain one similar and three identical amino acids and both sequences have the potential to form internal disulphide bonds. The peptides may therefore be able to adopt a putative loop structure, which would probably be rare in an unconstrained peptide. These disulphide bonds may be important in stabilising the conformation of the peptides, thereby allowing them to bind the paratope with sufficient affinity to be panned in comparison to a flexible linear peptide.

In ELISA, both fusion phages inhibited binding of Mab 4.52 to immobilised *Mycoplasma capricolum* subsp. *capripneumoniae* lysate (figure 3.4.8). This inhibition

was relatively inefficient, requiring high concentrations of fusion phage. Not surprisingly, the Mab therefore probably had a relatively lower affinity for the peptides than for its authentic epitope. An indication of the kinetics of dissociation of the Mab from a phage displayed peptide can be obtained from the sensorgram shown in figure 3.4.7. In this experiment the phages displaying peptide X2 were immobilised on the sensor surface. During the dissociation phase, there was a rapid decrease in the response level. By comparison, in the opposite orientation, there was a much slower dissociation of fusion phages from the immobilised Mab. This is probably the result of a multivalent interaction of the multiple copies of each fusion peptide displayed on the phage surface with the affinity matrix.

It is often possible to locate an antibody's cognate epitope by aligning sequences selected from a phage display library with the deduced amino acid sequence of the original antigen (Du Plessis *et al.*, 1994; Heiskanen *et al.*, 1999; Bentley *et al.*, 2000). At this time insufficient sequence data are available to make this possible with Mccp. It seems likely, in view of the disparate antigenic peptide sequences selected, that at least one or perhaps both, represent a mimotope, the sequence of which may be impossible to match completely. In any event, the peptide sequences were aligned with the available amino acid sequences of *Mycoplasma mycoides* subsp. *mycoides* SC, the causative agent of contagious bovine pleuropneumonia. Only peptide X1 showed significant alignment with the available sequence data. If an open reading frame can be identified in which the peptide sequence is mapped, the protein, which the gene encodes can be expressed and characterised, leading to possible diagnostic applications. If the Mab, however, recognises either a discontinuous epitope on the Mccp antigen or a non-proteinaceous epitope a simple match with a linear amino acid sequence is unlikely to be found.

Although they were recognised by Mab 4.52 in several different assays, neither peptide X1 nor X2 was detectable using a *Mycoplasma capricolum* subsp. *capripneumoniae*-specific goat antiserum. In contrast, this antiserum efficiently inhibits binding of Mab 4.52 to immobilised *Mycoplasma capricolum* subsp. *capripneumoniae* lysate and is used as a positive control in a standardised test for *Mycoplasma capricolum* subsp. *capripneumoniae* antibodies (Thiaucourt *et al.*, 1994). Keeping in mind that immunological interactions are natural phenomena, it is true that

the indications of antigenicity depends largely upon how it is measured (Van Regenmortel, 1989). The goat antiserum may therefore simply have had too low an affinity for the substitute antigens to produce a signal in indirect ELISA. It was also unable to compete with the Mab for binding to immobilised fusion peptide X2 even though surface plasmon resonance shows that the Mab dissociates relatively rapidly. Together these results suggest that the goat antisera may have in fact been completely devoid of paratopes that could accommodate the surrogate epitopes so that the binding shown in figure 3.4.11 was only due to non-specific binding to the dextran surface. This is substantiated by the quick dissociation phase of the antiserum on the immobilised phages. It is furthermore conceivable that goat antibodies do not recognise exactly the same antigenic site as the murine Mab itself. Inhibition of the Mab in the CCPP blocking ELISA would then depend on steric blocking by antibodies that bind to adjacent sites rather than a functional blocking. If so, goat antisera could not be expected to prevent the Mab from binding to a surrogate epitope outside the context of the original antigen. The surrogate antigens produced by the approaches investigated in this study depend entirely upon the characteristics of the original antibody. To develop useful immunoassays for CCPP diagnosis, it will therefore be necessary to investigate other Mabs, or possibly even polyclonal serum from an infected animal that has been suitably cross-absorbed, to yield antibodies of the desired specificity.

Chapter 4

Concluding remarks

If I have seen further, it is by standing on the shoulders of giants.

Isaac Newton, 1642-1727

At the beginning of this investigation a monoclonal antibody, Mab 4.52, recognising an epitope on *Mycoplasma capricolum* subsp. *capripneumoniae* cell lysate was chosen as a base to develop surrogate antigens displaying this epitope, to increase the specificity and sensitivity of CCPP diagnosis. The Mab was chosen from a panel of 60 Mabs, which were produced by Thiaucourt and co-workers (1994). In their attempts to find one with the desired specificity, only one, Mab 4.52 showed the most promise in a blocking ELISA. Although seemingly sufficient specificity for the diagnosis of CCPP, in this ELISA, cross-reactions with *Mycoplasma* species group 7 strains and one strain of *Mycoplasma capricolum* was observed. Nevertheless, it was postulated that by isolating an epitope recognised by Mab 4.52 rather than using the entire microorganism as antigen, a more effective ELISA could be developed.

Two cross-reactive antigenic peptide sequences could be selected from a phage display library, while a population of anti-idiotype antibodies was found to be present in an immunised chicken's egg-yolk IgY. The anti-idiotype antibodies had antigenic characteristics in common with the peptides since both types of substitute antigens inhibited binding of Mab 4.52 to *Mycoplasma capricolum* subsp. *capripneumoniae* lysate. However, the anti-idiotype IgY appeared to have a higher affinity for Mab 4.52's paratope than did peptide X2. No inhibition was evident when anti-Mab 4.52 IgY was immobilised to the solid-phase and the peptide competing for Mab 4.52, whereas the anti-Mab 4.52 IgY was able to compete with peptide X2 on the solid-phase for the Mab. This could be as a consequence of the immune system of the chicken generating a molecule, which has a higher affinity for the Mab 4.52 because of a better "fit" than the peptides selected. Perhaps the peptide and the anti-Mab 4.52 IgY recognise different regions of the paratope lying in close proximity and so appeared to be cross-reactive.

This study has shown that antigenic mimics recognised by a *Mycoplasma capricolum* subsp. *capripneumoniae* Mab can be obtained by the two different approaches. Each strategy has its advantages. The egg-yolk anti-idiotype approach is a simple technology; each generation of antibodies that is produced have a higher affinity for the antigen due to gene conversion (Higgins, 1996), but the requirement for complex immunoaffinity

purification would probably limit the amount of useable IgY which can be obtained in practice. In contrast, a major advantage of phage display is that it uses no animals and yields defined peptide sequences which can be synthesised in unlimited quantities. As long as the epitope is largely continuous, this could allow the gene encoding the entire antigenic protein to be located and thereby identify diagnostically important antigens. In conclusion, even though the goat antisera did not recognise any of the isolated surrogate epitopes, both peptide mimotopes identified by phage display and the chicken anti-idiotype antibodies generated, have elucidated some of the characteristics of the epitope recognised by Mab 4.52. It seems likely that it is not immunodominant in Mccp infections, as the antisera appear to be devoid of the corresponding paratope. Several alternatives can be investigated further, but the most promising and possibly the most interesting would be to select random peptides using polyclonal antiserum as the target. The antiserum should contain antibodies to all or most of the epitopes that the organism has been exposed to and this should allow several peptides to be selected from the display library. In order to enrich for disease-specific peptides, the undesirable phages and the phages that bind outside of the antigen binding site need to be subtracted. This can be done by using a pool of sera from uninfected goats. The phages that react with the Mccp antisera, but not with the sera from uninfected goats will be considered as relevant phages. This will allow specific phages to be selected without prior knowledge of the antibodies or the proteins involved. Alternatively, by screening peptide phage libraries with sera from animals which have recovered from the disease, peptides for protective antibodies may be identified and the recombinant phages use as a vaccine to induce a protective humoral response. The final sequencing of the complete *Mycoplasma mycoides* subsp. *mycoides* SC genome will also help identify gene sequences corresponding to the peptide sequences selected. The relevant proteins can then be expressed and investigated as diagnostic reagents or for their ability to protect against CCPP.

Appendices

1. Constituents of buffers, stock solutions and media

0.07 M Acetate buffer pH4

2.87 g sodium acetate
x ml deionised water
adjust pH to 4 with glacial acetic acid
---
500 ml final volume

10 mM Acetate buffer pH 4

1.6 g sodium acetate
x ml deionised water
adjust pH with glacial acetic acid
---
2 l final volume

30% Acrylamide

30 g acrylamide
x ml deionised water
---
100 ml final volume
filter and store at 4°C in the dark

Acrylamide/bis-acrylamide solution (38%/2%)

38 g acrylamide
2 g bis-acrylamide
x ml deionised water
---
100 ml final volume
filter and store in the dark at 4°C

10% Ammonium persulphate

0.1 g ammonium persulphate
x ml deionised water
---
1 ml final volume

Ammonium sulphate (saturated)

761 g ammonium sulphate
x ml deionised water
---
1 l final volume

5% Azide

0.05 g NaN_3
1 ml deionised water

2% BSA/TBS

2 g Bovine serum albumin
100 ml 1 X TBS
---
100 ml final volume

Destain solution I

200 ml methanol
50 ml acetic acid
250 ml deionised water
---
500 ml final volume

Destain solution II

100 ml methanol
50 ml acetic acid
250 ml deionised water
---
500 ml final volume

Destain solution III

2.5 ml methanol
497.5 ml deionised water
---
500 ml final volume

10% Developer

300 ml developer
2700 ml deionised water
---
3 l final volume

Elution buffer

1 ml 32% HCl
x ml deionised water
adjust pH to 2.2 with glycine
---
100 ml final volume

70% Ethanol

70 ml 99.9% ethanol
30 ml deionised water
---
100 ml final volume

25% Fixer

875 ml fixer
2625 ml deionised water

3.5 l final volume

LB-agar

4 g bacto-tryptone
4 g NaCl
2 g yeast extract
4.8 g agar
x ml deionised water
---
400 ml final volume

LB-broth

4 g bacto-tryptone
4 g NaCl
2 g yeast extract
x ml deionised water
---
400 ml final volume

2% Milk powder/PBS

2 g ELITE milk powder
x ml PBS
---
100 ml final volume

NAP buffer pH 7

160 µl 5 M NaCl
1 ml 0.5 M $NH_4H_2PO_4$
x ml deionised water
---
10 ml final volume
store at 4°C

PBS/0.05% Tween 20

500 µl Tween 20
1 l PBS

PBS/0.1% Tween 20

1 ml Tween 20
1 l PBS

PEG/NaCl (16%/3.3M)

100 g	PEG 8000
116.9 g	NaCl
x ml	deionised water
---	
1 l	final volume

2X PSB

2 g	sucrose
0.1 g	dithiotreitol
0.5 g	SDS
0.001 g	bromofenolblue
1.25 ml	1 M Tris pH 6.8
x ml	deionised water
---	
10 ml	final volume

4X Separating buffer

36.3 g Tris
0.8 g SDS
x ml deionised water
adjust pH to 8.8
---
200 ml final volume

3M Sodium acetate

61.52 g	CH_3COONa
x ml	deionised water

adjust pH to 6 by adding glacial acetic acid
---
250 ml final volume

5M Sodium chloride

29.22 g	NaCl
x ml	deionised water
---	
100 ml	final volume

4X Stacking buffer

12.1 g	Tris
0.8 g	SDS
x ml	deionised water
adjust pH to 6.8	
---	
250 ml	final volume

Substrate

0.01 g	OPD
10 ml	0.1 M citrate buffer
5 µl	30% H_2O_2
---	
10 ml	final volume

2 N Sulphuric acid

1 ml	concentrated H_2SO_4
17 ml	deionised water
---	
18 ml	final volume

50X TAE buffer

40 mM	Tris.HCl
20 mM	NaOAc
1 mM	EDTA
adjust pH to 8.5	

10X TBE

108 g	Tris
9.3 g	$Na_2EDTA.2H_2O$
55 g	boric acid
x ml	deionised water
---	
1 l	final volume

10X TBS

50 ml	1 M Tris pH 7.5
30 ml	5 M NaCl
x ml	deionised water
---	
1 l	final volume

TBS/0.05% Tween 20

250 µl	Tween 20
999.75 ml	1 X TBS
---	
1 l	final volume

TBS/0.5% Tween 20

500 µl	Tween 20
999.5 ml	1 X TBS
---	
1 l	final volume

TE buffer

0.61 g	Tris
0.19 g	Na_2.EDTA
x ml	deionised water
---	
500 ml	final volume

10 mg/ml Tetracycline

1 g	tetracycline
50 ml	deionised water
50 ml	99.9% ethanol
---	
100 ml	final volume
filter	

10X TGS

126.4 g	Tris
79.8 g	glycine
x ml	deionised water
adjust pH to 9.2	
---	
1 l	final volume

0.1 M Triethylamine solution pH 12

140 µl	triethylamine solution
10 ml	deionised water

1 M Tris pH 7.4 / pH 9.1

30.29 g	Tris
adjust pH to 7.4 or 9.1	
x ml	deionised water
---	
250 ml	final volume

7 M Urea

105 g	urea
x ml	deionised water
---	
250 ml	final volume

2. One-letter code for amino acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Feature	**Amino acid one-letter code**
Hydrophobic	HWYFMLIVCAGTK
Aliphatic	LIV
Aromatic	FYWH
Polar	TSNDEQRKHWY
Charged	DERKH
Positive	RKH
Small	PVCAGTSND
Tiny	AGS
Glycine	G
Proline	P

(Source: Nature Biotechnology, volume 16 1998)

3. Scientific contributions

- Manuscript submitted to *Veterinary Microbiology*

  Benguric D. R., Dungu B., Thiaucourt F. and Du Plessis D. H. Phage displayed peptides and anti-idiotype antibodies recognised by a monoclonal antibody directed against a diagnostic antigen of *Mycoplasma capricolum* subsp. *capripneumoniae.*

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