

Antigenic analysis of cowpea severe  
mosaic virus and other selected viruses using  
monoclonal antibodies

by

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Signatures have been redacted for privacy

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## TABLE OF CONTENTS

	Page
INTRODUCTION.....	1
LITERATURE REVIEW.....	6
Cowpea Severe Mosaic Virus.....	6
Cowpea Mosaic Virus.....	16
Beanpod Mottle Virus.....	17
Maize Dwarf Mosaic Virus.....	18
Soybean Mosaic Virus.....	18
Lettuce Mosaic Virus.....	19
Barley Stripe Mosaic Virus.....	19
Monoclonal Antibodies.....	20
Immunoassays.....	25
MATERIALS AND METHODS.....	36
Virus Propagation.....	36
Purification of Cowpea Severe Mosaic Virus.....	36
Purification of Cowpea Mosaic Virus.....	38
Purification of Beanpod Mottle Virus.....	39
Sources of Other Viruses.....	41
General Methods.....	41
Distilled and deionized water.....	41
Production of Monoclonal Antibodies.....	41
Dulbecco's modified eagle medium (DMEM).....	42
Growth and maintenance media (DMEM-R).....	43
Myeloma cells.....	43
8-Azaguanine media.....	43
Conditioned media.....	44

HAT media.....	44
HT media.....	45
Freezing media.....	46
Mouse injection schedule.....	46
Production and maintenance of hybridomas.....	46
Ascites fluid production.....	49
Indirect ELISA.....	49
Coating buffer for virus.....	50
Wash buffer (PBS-Tween; 20X).....	50
Blocking buffer (Blotto).....	51
Substrate buffer.....	51
Preparation of substrate.....	52
Optimization of the indirect ELISA.....	52
Indirect ELISA assay.....	53
Determination of Immunoglobulin Class and Subclass.....	54
Purification of Monoclonal Antibodies from DMEM-R Media.....	55
The Antigenic Analysis of Comoviruses and Other Antigens, and the Characterization of Monoclonal and Polyclonal Antibodies by Immunoblotting.....	56
Viruses, other antigens and antibodies utilized in immunoblotting.....	56
Disruption of viral proteins.....	57
SDS polyacrylamide gel electrophoresis.....	58
Immunoblotting procedure.....	64
Modifications of immunoblot assays.....	69
Serologically Specific Electron Microscopy.....	69
Characterization of Monoclonal Antibodies by Competition Assays.....	70
Biotinylation of antibodies.....	71
Competition assay procedure.....	71
RESULTS.....	76
Production of Monoclonal Antibodies.....	76

Optimization of Indirect ELISA.....	76
Isotyping of Monoclonal Antibodies.....	77
Purification of Antibodies.....	78
Immunoblot Analysis.....	78
Electron Microscopy Studies.....	81
Competition Assays.....	81
DISCUSSION.....	110
SUMMARY.....	144
LITERATURE CITED.....	146
ACKNOWLEDGEMENTS.....	161

## INTRODUCTION

Murine monoclonal antibodies have been used extensively in immunological assays since methods for producing antibody secreting hybridoma cell lines were first developed by Kohler and Milstein (1976). They offer several advantages over polyclonal antibodies such as: significantly smaller quantities of antigen are required for immunization, the specificity of monoclonal antibodies is greater than polyclonal antibodies and, it is easier to standardize assays using monoclonal antibodies because a consistent source of antibody is available once a cell line has been established. Hybridoma technology has only been utilized by plant virologists for a short period of time; however, it has already demonstrated its usefulness (Van Regenmortel, 1984).

Clark and Adams (1977) were the first to utilize the ELISA for the study of plant viruses. Since that time, it has been used extensively. Various types of ELISAs exist (Barbara and Clark, 1982; Koenig and Paul, 1982). The double antibody sandwich ELISA is used most frequently in plant virology (Lommel et al., 1982). However, the indirect ELISA is also a sensitive assay that can be used for the detection of plant viruses and the selection of

involved in monoclonal antibody producing cell lines.

Competition analysis can be utilized to characterize monoclonal antibodies and the sites to which they specifically bind. A labelled and an unlabelled antibody are used in these assays. In enzyme-linked immunosorbent assays (ELISA) the labelled antibody can be conjugated with biotin; then, avidin conjugated with an enzyme is added followed by an appropriate enzyme substrate and chemogen and the assay is read spectrophotometrically. The ability of the unlabelled antibody to compete with the biotinylated antibody can be monitored by observing a decrease in optical density.

Monoclonal antibodies should be characterized so that those antibodies that have desired characteristics can be identified and therefore selected for various analyses such as diagnostic assays. Immunoblot analysis is one method of characterizing antibodies. In this system, proteins are disrupted, resolved electrophoretically on a polyacrylamide gel, transferred to nitrocellulose and probed with a panel of antibodies. The binding pattern of each of the antibodies is one of its "characteristics". The antigenic relationships of viruses and other antigens can also be resolved by immunoblot assays. Determining whether antigens are related is of importance because relatedness would indicate that some antibodies can react

with antigens that have been identified as having common antigenic determinants. Since antibodies are frequently employed in diagnostic assays for identifying the causal agent of a disease, it is important to know whether they are specific for the causal agent or whether they also react with closely related or supposedly unrelated antigens. Specific antibodies can be selected by testing a series of different antibodies against a panel of antigens that are known to be related and determining which of the antibodies reacts only with the causal agent. As suggested, ascertaining which antigens should be included in this type of assay, can be determined by immunoblot analysis.

The original objective of this research was to produce monoclonal antibodies to cowpea severe mosaic virus serotype II (CPSMV II) and to characterize these antibodies as well as monoclonal antibodies previously produced against CPSMV I (provided by Dr. J. H. Hill, I.S.U., Ames, IA). This was attempted by testing them against several comoviruses utilizing immunoblot analysis. However, because no differences were observed in the antibodies in these assays, the antibodies were also tested against four other plant viruses from two plant virus groups, one animal virus and other proteins. The plant viruses utilized were selected because they are

in different plant virus groups (potyvirus and hordeivirus groups) and were thought not to be serologically related to the comoviruses. The other antigens were chosen for the same reasons; i.e., they were assumed not to be serologically related to the comoviruses. Difficulties in characterizing the monoclonal antibodies continued to exist, even when such a broad array of antigens was utilized since the majority of the antibodies reacted with many of the viruses and proteins. These results indicated that it was possible that these antigens may have had common epitopes. As a result, more assays were conducted, but with a larger pannel of antibodies that included monoclonal and polyclonal antibodies produced against other viruses, with the intent of not only characterizing the antibodies but also determining whether the antigens shared common epitopes. Since many of the antibodies continued to react with numerous viruses and other antigens, characterizing the antibodies and antigens remained equivocal. Therefore, an alternative approach, which involved competition assays, was employed for characterizing some of the antibodies against one of the antigens (CPSMV I). This was a preliminary study to identify the monoclonal antibodies which differed the most in relation to how they reacted specifically with CPSMV I. Selected antibodies could then be utilized, in the



future, to determine the topographical arrangement (which is the demonstration of antigenic differences between proteins, Yewdell and Gerhard, 1981) of antigenic sites of different serotypes of CSPMV. Differences in their epitope topology could potentially be correlated to the phenotypic expression of virus infectivity in different hosts by the virus serotypes. This information could then be utilized by plant breeders in the development of resistant hybrids. Furthermore, assays could be performed every few years to determine whether antigenic drift has occurred in the interim years. Antigenic drift would indicate that new serotypes of the virus may have evolved that may be capable of overcoming resistant hosts and this may signify that new resistant varieties should be developed. Knowledge of the topography of antigenic determinants of serotypes of CSPMV could also aid in future studies of virus-vector relationships.

## LITERATURE REVIEW

## Cowpea Severe Mosaic Virus

Cowpea severe mosaic virus (CPSMV) is a member of the comovirus group. These are multicomponent viruses that consist of three icosohedral particles which are referred to as the top, middle and bottom components. Originally CPSMV was considered to be one of the two subtypes of cowpea mosaic virus (CPMV). In 1979, CPMV was separated into two viruses: CPSMV and CPMV which were formerly the severe and yellow subtypes of CPMV, respectively (de Jager, 1979). They were considered to be two different viruses on the basis of the following factors: host range, antigenic properties, host reactions, heterology in nucleotide sequences and inability of heterologous nucleoprotein components to complement one another (Swans and van Kammen, 1973). For example, in contrast to CPMV, CPSMV is unable to infect Chenopodium amaranticolor systemically. Furthermore, CPSMV has a higher middle to bottom component ratio than CPMV (de Jager, 1979).

CPSMV has several synonyms: cowpea mosaic virus, severe subgroup (Thongmeearkom and Goodman, 1978), Arkansas mosaic virus (Shepherd, 1964), and Trinidad cowpea

mosaic virus (Chant, 1962).

The geographical distribution of this virus includes Trinidad, Puerto Rico, the U.S.A., El Salvador, Costa Rica, Venezuela, Surinam, Brazil and Peru (de Jager, 1979). Many leguminous crops and weeds such as Chenopodium amaranticolor (lamb's-quarters), Phaseolus (bean) and Vigna unguiculata (cowpeas) may be infected. Its most significant economic impact is on cowpeas. Dale (1949) recovered CPSMV in as much as 100% of the plants examined. A reduction in the number of pods and fresh weight of cowpeas infected with CPSMV was observed by Debrot and de Rojas (1967). Soybeans (Thongmeearkom and Goodman, 1976) and other leguminous crops (Dale, 1949) have been severely infected as well. Diagnostic species include Chenopodium amaranticolor, on which small necrotic lesions are produced on inoculated leaves, and Vigna unguiculata cv. Blackeye Early Ramshorn, where veins become necrotic and leaves develop chlorotic and necrotic lesions. This cowpea cultivar is also good for maintenance and as a source for viral purification (de Jager, 1979).

CPSMV can be transmitted mechanically, through seeds or by leaf feeding beetles primarily from the Chrysomelidae insect family. The virus can be acquired by the beetles after they have only been feeding for five

minutes and they can transmit the virus for up to two weeks after uptake (Dale, 1953; Walters and Barnett, 1964). The virus was identified in the haemolymph (Sanderlin, 1973) and regurgitant of beetles (Dale, 1953) after injection or natural uptake of the virus (Slack and Fulton, 1971). The transmission efficiency and persistence of the virus is directly correlated with the acquisition period (Dale, 1953; Jansen and Staples, 1971).

Various isolates of the virus have different rates of seed transmission. Shepherd (1964) found that 10% of cowpea seeds became infected with the Arkansas isolate. The Trinidad isolate was transmitted to 8% of the asparagus bean seeds tested (Dale, 1949).

Amorphous inclusion bodies have been observed in the proximity of and surrounding the nucleus in epidermal strips of cowpeas and peas. These strips were stained with phloxine and studied with a light microscope (Swans and van Kammen, 1973).

Debrot and de Rojas (1967) concluded that virus from the primary leaves of Pinto beans infected with CPSMV had a thermal inactivation point between 65 and 70 C, a dilution end point ranging from  $10^{-4}$  to  $10^{-5}$  and a longevity in vitro of one to five days at a temperature of 24 C.

CPSMV, as other comoviruses, produces three icosohedral particles of approximately 28 nm which can be

separated on a sucrose gradient and are referred to as the top (T), middle (M) and bottom (B) components. The top component is solely an empty protein shell whereas the latter two are nucleoproteins (Goldbach and van Kammen, 1985). The RNAs of the middle and bottom components have different molecular weights (Thongmeearkom and Goodman, 1978); however, their capsid proteins are identical morphologically and serologically (Shepherd, 1964).

The morphology and function of the capsid proteins and RNA of comoviruses has been studied most extensively with cowpea mosaic virus (CPMV). For this reason, the following information will emphasize research conducted in these areas with CPMV and not CPSMV.

The capsid of CPMV and other comoviruses consists of one large and one small protein. These proteins can be separated by polyacrylamide gel electrophoresis into three bands; one major and two minor bands which correspond to the large and small proteins, respectively (Lin et al., 1984; Stace-Smith, 1981; Thongmeearkom and Goodman, 1978). The two minor bands are considered to be size isomers of the same protein and are referred to as the slow and fast forms. Niblett and Semancik (1969) stated that the fast form may be produced when three amino acids are lost at the C-terminus of the slow form of the minor band. Geelen et al. (1972) also observed that the fast

form was derived from the slow form but that there was a 2500 dalton difference between the two forms rather than just three amino acids. The conversion of the small protein of CPMV from the slow to fast polypeptide is thought to be due to host proteolytic enzyme action (Niblett and Semancik, 1969). Similar conclusions have been reached with CPSMV (Thongmeearkom and Goodman, 1978).

The amount of slow versus fast protein varies depending upon several factors such as: the comovirus, the time after inoculation that the virus is purified from the plant, the method of disruption of the virus and the reagents used in electrophoresis. For example, the ratio of the slow to the fast form increases with time after inoculation of the related bean pod mottle virus (BPMV) on soybeans (Glycine max (L.)) (Bancroft, 1962).

The molecular weights of the large and slow and fast forms of the small proteins of CPMV were estimated to be 44,000, 25,000 and 22,000 daltons for carboxymethylated proteins, and, 49,000, 27,500 and 25,000 daltons for oxidized proteins (Geelen et al., 1972). Under conditions used by Wu and Bruening (1971), the molecular weights of the large and small proteins were 37,000 and 23,000 daltons. In this situation the molecular weights of both forms of the small protein were not differentiated. The molecular weights of the large and small proteins of CPSMV

are 40,300-41,800 and 20,100-21,400 daltons respectively (Lin et al., 1984). The possibility of a monomer-dimer system has been contemplated because of the ratio of the molecular weights of the proteins. However, it seems that this relation does not exist because non-hydrolytic degradation procedures did not change the molar ratios of the proteins. Moreover, determinations of the amino acid compositions disproved the possibility that the large protein was in fact a covalently linked dimer (Geelen et al., 1972).

As stated previously, CPMV consists of three icosohedral particles. The middle and bottom particles contain positive sense single-stranded RNA whereas the top component is an empty protein shell (Goldbach and van Kammen, 1985). The molecular weights of the bottom and middle RNA (B-RNA and M-RNA) are  $2.04 \times 10^{-6}$  and  $1.22 \times 10^{-6}$  daltons, respectively (Ahlquist and Kaesberg, 1979). Both RNAs have a small Vpg (viral protein genome-bound) protein bound to their 5' ends and a polyadenylated sequence at their 3' ends (Goldbach et al., 1981).

The translation strategy of CPMV is complex. The primary product of B-RNA is a 200 K polyprotein which is cleaved to form a 32 K and 170 K polypeptides. The 170 K protein is, in turn, proteolyzed to 60 K and 110 K, or 84 K and 87 K proteins. The 60 K polypeptide is cleaved to

form a 58 K and Vpg (4 K) proteins; the 110 K polypeptide into 28 K and 87 k proteins; and the 84 K polypeptide to 58 K, Vpg (4 K) and 28 K proteins. The 87 K polypeptide is not reduced to smaller proteins. The primary translation products of M-RNA are 105 K and 95 K polyproteins. These proteins can either be proteolyzed to a 93 K, 79 K and two VP (viral protein) 23 proteins, or a 58 K, 48 K, and two 60 K proteins. With respect to the latter cleavages, the 60 K proteins are cleaved to 37 K and 23 K proteins which are the capsid proteins (Goldbach and van Kammen, 1985).

Middle and bottom RNA are both necessary for virus multiplication whether they are from the same virus or from different mutants (Thongmeearkom and Goodman, 1978). B-RNA can replicate and synthesize B-RNA encoded polypeptides independently of M-RNA but cannot become systemic in plants and produce symptoms (de Jager, 1976). This is because M-RNA is responsible for capsid protein production and encapsidation, and cell-to-cell transport of viral RNA (Rezelman et al., 1982). B-RNA encodes proteases which are involved in processing the primary translation products of both B-RNA and M-RNA (Wellink et al., 1986). M-RNA is incapable of replicating on its own and is therefore dependent upon proteases encoded by B-RNA (Franssen et al., 1982).



Serological relationships and therefore shared antigenic determinants have been demonstrated to exist among four serotypes of CPSMV, plant viruses among the same virus family, the large and small proteins of squash mosaic virus (SqMV; a comovirus), plant viruses from different virus groups, and unrelated molecules. Lin et al. (1984) conducted serological studies to determine the antigenic relationships of serotypes I, II, III and IV of CPSMV. Relationships were evaluated by a comparison of titers of the antisera and spur formation in immunodiffusion tests. The titers of the antibodies indicated that serotypes I, III and IV were more closely related to each other than to serotype II. They concluded that immunodiffusion assays showed that serotypes I and IV shared three epitopes; I, III and IV had two common epitopes; and, I, II, III and IV only shared one epitope. Therefore, I and IV are most closely related and II the least related to the other serotypes. The results from these two assays indicate that a certain amount of antigenic relatedness does exist among all four serotypes of CPSMV.

Studies have also been conducted to determine the antigenic relationships between different comovirus. Swans and van Kammen (1973) performed studies to determine the antigenic relationships between CPMV and CPSMV both of which are comoviruses. They found a weak immunological

relationship between these viruses. Trinidad and Arkansas CPMV, now considered two serotypes of CPSMV (de Jager, 1979), were found to be related to each other and more distantly to BPMV. Two-dimensional double immunodiffusion, tube precipitation and cross-absorption were used to confirm these relationships (Shepherd, 1964). This corroborated results published earlier by Shepherd (1963) in which the serological relationships of many viruses such as Arkansas and Trinidad CPMV, BPMV, Nigerian cowpea yellow mosaic virus, tomato ringspot virus, cucumber mosaic virus and the cowpea strain of southern bean mosaic virus were studied. The first three viruses displayed antigenic relatedness. Five isolates of CPMV were compared by Agrawal and Maat (1964). Three of these isolates were considered to be cowpea yellow mosaic virus (now CPMV) and the others were the severe subgroup of CPMV (now CPSMV). All of these isolates shared antigenic determinants with each other, and, with BPMV and red clover mottle virus. Slack and Fulton (1971) stated that CPMV, BPMV and SqMV, all of which are comoviruses, are related serologically. Pea mild mosaic virus and Trinidad CPSMV were reported to be related by Clark (1972). CPMV cross-reacted with bean rugose mosaic virus (Gamez, 1972) and broad bean true mosaic virus (Jones and Barker, 1976).

The antigenic relationships of the large and small

proteins of comoviruses have also been studied. Hiebert and Purcifull (1981) produced polyclonal antibodies to both the small 22 K and the large 42 K coat proteins of SqMV. These antibodies were tested, by immunoprecipitation, against the products translated from the M-RNA and B-RNA of SqMV. The antisera produced against the 22 K protein reacted with all of the products translated from M-RNA which included the 22 K and 42 K coat proteins. Antisera generated against the 42 K coat protein reacted with all products that were equal to or larger than 36 K. These results suggest that there is a certain amount of antigenic homology with respect to the small and large coat proteins of SqMV. Since this virus is also related serologically to BPMV and CPMV (Slack and Fulton, 1971), and antisera to CPMV reacts with CPSMV (Agrawal and Maat, 1964), the evidence suggests that the small and large proteins of these comoviruses may also contain common epitopes. Wu and Bruening (1971) digested the large and small proteins of CPMV and compared a composite pattern of ninhydrin-positive spots and found nine common spots for the large and small proteins. This suggests that a certain degree of homology exists between the two proteins of CPMV. All the evidence presented above indicates that many of the comoviruses share some antigenic determinants and that the potential also exists that their small and

large capsid proteins do as well.

CPMV has already been discussed in detail so that comparisons between it and CPSMV could be made. However, important properties of CPMV as well as other viruses that were studied have also been summarized below.

#### Cowpea Mosaic Virus

CPMV is a member of the comovirus group and produces three icosohedral particles: the top, middle and bottom. Each has a diameter of 28 nm (Goldbach and van Kammen, 1985). The top component contains no RNA whereas the middle and bottom particles each contain positive sense single-stranded RNA (de Jager, 1976; van Kammen, 1968). As with CPSMV, it infects primarily plants in the family of Leguminosae. Infected cowpeas display a variety of symptoms ranging from a slight green mottle to a severe mosaic. Other hosts may develop chlorotic and necrotic lesions (Bliss and Robertson, 1971). Beetles transmit the virus although it can also be transferred mechanically (Chant, 1959; Jansen and Staples, 1971). Thrips and grasshoppers are also vectors but they do not transmit the virus efficiently (Whitney and Gilmer, 1974). Seed transmission may occur as well (van Kammen and de Jager, 1978).

The capsid consists of two proteins. There are sixty

copies of each protein in the capsid (Geelen et al., 1972; Wu and Bruening, 1971). These coat proteins can be resolved by gel electrophoresis into two or three bands (van Kammen and de Jager, 1978).

Two of the three viral particles contain RNA. Vpg is found at the 5' terminus and the 3' end is polyadenalated (Ahlquist and Kaesberg, 1979; El Manna and Bruening, 1973). The translational strategy and function of both RNA strands within the middle and bottom components have been previously discussed.

#### Beanpod Mottle Virus

BPMV, a member of the comovirus group, is very similar to CPSMV and CPMV. This virus produces three icosohedral particles (top, middle and bottom) which each have a diameter of 30 nm (Semancik and Bancroft, 1965). The middle and bottom particles contain single-standed RNA (Bancroft, 1962). The capsid is composed of two proteins which have molecular weights of 39,000 and 20-22,000 daltons respectively. The viral proteins can be separated by gel electrophoresis into three bands (Lin and Hill, 1983).

BPMV primarily infects plants of the family Leguminosae and also Chenopodium quinoa (Lin and Hill,

1983; Moore and Scott, 1971). Propagation and diagnostic species include Phaseolus vulgaris and Glycine max. Symptoms of chlorosis, necrosis and mottle can occur and are dependent upon the host and environment (Semancik, 1972). Beetles are vectors for transmission (Patel and Pitre, 1971; Walters, 1964, 1969). Lin and Hill (1983) indicated that BPMV can be transmitted by seed.

#### Maize Dwarf Mosaic Virus

Maize dwarf mosaic virus (MDMV) is a member of the potyvirus group. Viral particles consist of flexous filamentous particles which enclose a single-stranded positive sense RNA molecule (Hollings and Brunt, 1981). This virus infects many species of Graminae but is most economically significant in maize (Rosenkranz, 1981).

#### Soybean Mosaic Virus

The potyvirus group also includes soybean mosaic virus (SMV). Viral particles are long flexous rods which contain positive sense, single-stranded RNA. This pathogen can cause important problems in soybeans throughout the world. SMV reacts serologically with bean yellow mosaic and bean common mosaic viruses (Edwardson,

1974; Ross, 1967).

#### Lettuce Mosaic Virus

Lettuce mosaic virus (LMV) is a member of the potyvirus group. Virus particles are filamentous and contain positive sense single-stranded RNA. This virus has a large host range but is significant economically on lettuce (Costa and Duffus, 1958). Sugarcane mosaic and turnip mosaic viruses may be distantly related to LMV (Brandes and Berks, 1965). Monoclonal antibody L5, which was produced to LMV, did cross-react with SMV, MDMV strain Ap and MDMV strain B (Hill et al., 1984).

#### Barley Stripe Mosaic Virus

Barley stripe mosaic virus (BSMV) is a member of the hordei family of plant viruses. It consists of short tubular particles containing single-stranded positive sense RNA (Atabekov and Novikov, 1971). Barley and wheat are natural hosts of BSMV. Chlorosis, necrosis and mosaic symptoms that can develop, are dependent upon the host and environmental factors (McKinney and Greeley, 1965). Serological cross-reactivity with lynchnis ringspot virus has been observed (Gibbs et al., 1963).

## Monoclonal Antibodies

Monoclonal antibodies have been used extensively in immunological studies since they were first developed by Kohler and Milstein (1976). Myeloma cells and spleen cells from immunized mice are fused and hybridoma cells are selected that produce antibodies specific to the antigen that the mice were injected with. These hybrid cells are selected and cloned until antibody secreting cell lines derived from single cells are established which produce monoclonal antibodies. These antibodies may complement polyclonal antibodies for the identification of viruses and in disclosing serological differences or similarities among viruses and other organisms.

The theory of monoclonal antibodies developed from the hypothesis of clonal selection (Burnet, 1959). This theory states that a repertoire of antigen-binding cells, existing within organisms capable of immune responses, are able to bind to specific antigens. When stimulated they evolve into plasma cells which produce antibodies specific to the antigens they were exposed to.

The idea that some cells are capable of secreting only one antibody grew from the observation that myeloma tumors produce only one type of immunoglobulin (Edelman et al., 1969). Potter (1972) and Cohn (1967) induced tumors



in mice by injecting mineral oil into the peritoneal cavity of mice. They observed that the tumors could be passed to other mice and that they could therefore have a continuous supply of antibodies of a single specificity. The difficulty with this strategy was that one could not control the specificity of the antibodies produced by the tumor cells. Their usefulness was therefore limited.

The transformation of B-cells by exposure to viruses has also been attempted. Fahler et al. (1966) transformed human lymphocytes with Epstein-Barr virus, and Abelson and Rabstein (1970) used Abelson virus to transform mouse lymphocytes. The difficulties inherent in this technique include: the diversity of antibodies produced to antigens is limited and only small yields of antibodies are obtained (Yelton and Scharff, 1981). Human blood cells were fused with mouse lymphoma cells by Schwaber and Cohen (1973). The fused cells secreted both human and mouse antibodies and therefore some specificity was lost. These experiments led to the development of hybridoma technology by Kohler and Milstein (1976).

Monoclonal antibodies can offer several advantages over polyclonal antibodies. (1) Significantly smaller quantities of antigen are required for immunization. If polyclonal antisera is produced in rabbits, milligrams of antigen are often utilized for immunization whereas only

micrograms are necessary when mice are injected for hybridoma development. (2) The specificity of monoclonal antibodies is greater than that of polyclonal antibodies. Polyclonal serum contains antibodies that can react to an array of antigens whereas monoclonal antibodies react with one antigen or antigens with similar epitopes. As a result, both similarities and differences among antigens are more easily recognized using monoclonal antibodies (Yelton and Scharff, 1981). (3) One of the problems posed in assays utilizing polyclonal antisera is the lack of standardization. The sera produced by different animals and even different batches from the same animal can vary. This can cause problems in obtaining reproducible results in research and diagnostic assays. On the other hand, once a hybridoma that produces a specific antibody has been established, a constant source of antibody is available.

The utilization of hybridoma technology is not always appropriate. The development of monoclonal antibodies is time consuming, requires a higher level of expertise, and is expensive. Furthermore, even if a useful antibody has been found, its specificity can be modified by changes in the pH (Mosmann et al., 1980), ionic strength (Haskell et al., 1983), temperature (Mosmann et al., 1980) and buffer salts (Pisetsky and Semper, 1984). Changes in any of the

above parameters can have an impact upon the sensitivity of an assay (Kammer, 1983), the affinity between antigens and antibodies, and cross-reactivity (Mosmann et al., 1980). Moreover, mutations in genes affecting antibody production may occur within a clone (Bruggemann, 1982) and the specificity of antibodies may also change if ascites fluid is produced (Bosch et al., 1982). One must therefore compare the advantages with the disadvantages to assess whether the production of monoclonal antibodies is appropriate for the desired assays.

The technique of somatic cell hybridization has been used extensively in medical and veterinary research. However, only since the early 1980s has it been used widely in plant virus research. Since that time, monoclonal antibodies have been produced to at least twenty plant viruses. In general, plant viruses are good antigens. This is probably due to the fact that they are unrelated to most proteins found within the mammalian system into which they are injected (Van Regenmortel, 1984).

Immunological assays are a critical factor in the identification of viruses as the causal agent of a plant disease. Plants infected with virus display symptoms such as chlorosis, necrosis and yellowing. These symptoms may also be caused by many other biotic and abiotic factors

such as: mineral deficiencies, air pollution, herbicides, insects, nematodes, and bacterial and fungal infections. For example, sucking insects can initiate necrotic lesions; however, fungal pathogens and viruses can as well. Since it is so difficult to know conclusively that a virus is the causal agent of a disease, serological assays are extremely useful in plant virology.

Polyclonal antiserum has been used for a long time to identify plant viruses. Ouchterlony tests were utilized by Lin and Hill (1983) to aid in the characterization of BPMV. Fulton and Scott (1979) also used this assay to reclassify the legume comoviruses. The ability of beetles to transmit squash mosaic virus and the cowpea strain of southern bean mosaic virus was studied by inoculating plants mechanically with beetle haemolymph. Ouchterlony tests were performed to verify that the healthy plants became infected. Polyclonal antibodies were also utilized by Hiebert and Purcifull (1981) to study the translational strategy of squash mosaic virus.

Monoclonal antibodies are increasingly being utilized in similar assays. Diaco et al. (1986) was able to detect barley yellow dwarf isolates using monoclonal antibodies in serologically specific electron microscopy. Carnation etch ring virus was detected by monoclonal and polyclonal antibodies in ELISAs and immunoelectron microscopy (Hsu

and Lawson, 1985). The antigenic structure of tobacco mosaic virus was studied using nine different monoclonal antibodies (Moudallal et al., 1982). Serotypes of tobamoviruses were differentiated with these antibodies by Briand et al. (1982).

The usefulness of hybridoma technology has already been demonstrated in plant virology and will continue to be in the future. Monoclonal antibodies will be extremely useful in comparing relationships among plant viruses and perhaps even between plant and animal viruses. The study of the structure and function of proteins may also be enhanced by using these immunoglobulins.

#### Immunoassays

Serological assays are useful for the diagnosis and study of plant viruses. Numerous assays exist which offer different advantages (Barbara and Clark, 1982; Kimball, 1983; Koenig and Paul, 1982). The primary advantage of some tests, such as the Ouchterlony double diffusion test, is that they are easy to perform. Others, such as rocket immunoelectrophoresis, offer speed (Kimball, 1983). This assay can be completed within hours instead of days required to obtain results in the Ouchterlony test. Immunofluorescence, which utilizes a fluorescent antibody to

detect antigens or antibodies, is qualitative. On the other hand, immunoblotting offers great sensitivity and simplicity. The advantages of radioimmunoassays are: one can assay many samples at one time, they are sensitive and quite easy to perform. The ELISA offers the advantages of radioimmunoassays but not the disadvantage of exposure to radioactive substances (Kimball, 1983). Furthermore, the utilization of many of the serological techniques mentioned above is limited for the detection of plant viruses due to factors such as the presence of viral inactivators or inhibitors in plant extracts, low virus concentrations and inappropriate morphology of virus particles. For example, rod shaped virus may not diffuse well in immunodiffusion assays because of their shape. The ELISA can often overcome these problems and also offer several additional advantages. This assay is quite simple to perform, is relatively fast, and it can be used for both qualitative and quantitative purposes (Clark and Adams, 1977).

Clark and Adams (1977) were the first to utilize the ELISA for the study of plant viruses. Since that time it has been used extensively by plant virologists. Shirako and Ehara (1985) were able to detect northern cereal mosaic virus in fresh, frozen, or freeze-dried diseased leaves, viruliferous planthoppers and purified virus at

concentrations down to 10 ng/ml. Tobacco ringspot virus and MDMV were identified in leaf discs and leaf homogenate by Romaine et al. (1981).

Various types of ELISAs such as the double antibody sandwich and the indirect ELISA exist (Barbara and Clark, 1982; Koenig and Paul, 1982). The double antibody sandwich ELISA is used most frequently in plant virology (Lommel et al., 1982). Two types of this assay exist: the indirect and the direct double antibody sandwich ELISA. With the latter an antibody is first adsorbed onto a well of a microtitre plate; then, virus is added, followed by an enzyme-conjugated antibody and then substrate appropriate to the enzyme. The enzyme-conjugated antibody remains in the well only if the virus is specifically bound by the primary antibody. In this case, the enzyme-linked antibody binds to the virus and reacts with the enzyme substrate. If this occurs, a color develops and the absorbance can be read spectrophotometrically (Clark and Adams, 1977). A similar protocol is followed in the indirect double antibody sandwich ELISA except for the following modifications. Two antibodies are added sequentially after the virus. The first one amplifies the sensitivity of the assay and the second one is the detection antibody since an enzyme is conjugated to it. The disadvantage of the double antibody sandwich ELISA is that

for each antigen that is assayed, antibodies must be purified and conjugated to an enzyme so that a specific enzyme conjugate is available for the antigen. This can be very time consuming. Moreover, this assay may be too specific. This can prevent detection of virus strains that are closely related (Van Regenmortel and Burkard, 1980). Therefore, it is sometimes preferable to use the indirect ELISA.

The indirect ELISA is a sensitive assay that can be used for the detection of plant viruses. In this assay, the antigen to be tested is bound directly to the solid phase and then an appropriate specific antibody is added. This is followed by an immunoglobulin specific conjugated antibody and appropriate substrate. This assay has several advantages over the double antibody sandwich ELISA. (1) Antibodies do not need to be purified and conjugated to an enzyme for each antigen that is to be detected. (2) Commercially available second antibodies can be utilized which obviates the storage and preparation of many different conjugated antibodies. A disadvantage of this assay is that it is difficult to quantitate viruses in crude plant extracts. This problem exists because host proteins and viruses compete for sites on the polystyrene surfaces of the ELISA plates (Lommel et al., 1982).

In addition to using the indirect ELISA for the



detection of plant viruses it can also be utilized for the selection process involved in monoclonal antibody production. The protocol used in these two assays is similar. Media from hybridoma cells growing in tissue culture plates is transferred to ELISA plates that have been coated with virus and have been blocked. Alkaline phosphatase conjugated goat anti-mouse IgM or IgG and substrate are added and the ELISA plate is read spectrophotometrically. Wells on the tissue culture plates that contained antibodies specific for the virus can be identified.

Immunoblot analysis can be utilized to characterize antibodies and to determine the antigenic relationships of proteins. In this assay proteins can be resolved electrophoretically on polyacrylamide gel, transferred to nitrocellulose and tested with a panel of antibodies. This protocol originated from the system developed by E. M. Southern (1975) who transferred DNA from agarose gel to nitrocellulose. The transfer not only of DNA but also RNA and protein from gels to solid matrices has been used extensively since then.

Until the development of these transfer procedures, it was difficult to infer the nature of bands on gels. For example, in immunoelectrophoresis, antigens within a gel were detected by allowing antibodies and the antigens

to diffuse towards one another. If a precipitate formed, specificity existed. This was a time consuming procedure and resolution was decreased because of diffusion of the proteins within the gel. Furthermore, the type of antigen and antibody, their concentrations and pore size of the gel were all critical and limited the use of immunoelectrophoresis. These difficulties were overcome by eluting proteins from gels to solid matrices (Towbin et al., 1979).

Proteins can be eluted by diffusion (Bowen, 1980), mass flow of solvents (Southern, 1975) or electroelution (Towbin et al., 1979). A gel that is to be eluted by diffusion is placed within two pieces of filter paper which are sandwiched between foam pads and screens. This is all placed in buffer for 36-48 hours. Protein from the gel is transferred to both sheets of filter paper. The limitations of immunodiffusion are that fairly lengthy transfer periods are required and only a limited amount of protein is transferred from the gel.

Southern (1975) described the transfer of DNA by mass flow. This can also be applied to proteins. A filter and a stack of paper towels are placed on a gel which in turn is placed in a chamber containing buffer. The buffer is absorbed into the towels and the protein is eluted from the gel into the filter.

Electroelution of proteins was developed by Towbin et al. (1979). One of the primary advantages of this technique is speed. Proteins are frequently transferred within three hours and occasionally transfer can occur in as little as one hour. The transfer system is assembled in the following manner. Nitrocellulose is placed on top of the gel. Both in turn are sandwiched between two pieces of filter paper, foam pads and perforated plastic sheets. This entire sandwich is placed within a transfer apparatus and the elution of proteins occurs.

Once proteins have been transferred from gel to nitrocellulose their antigenic relationships can be determined by probing them with a panel of antibodies, and concurrently, the antibodies that are being used as probes can be characterized by observing which antigens they bind to (their binding pattern). This is accomplished by incubating the nitrocellulose with a blocking agent (to diminish non-specific reactions), and then with antibodies (primary antibodies) which bind to all proteins that have epitopes that the antibodies are specific for. An enzyme conjugated antibody (secondary antibody), such as alkaline phosphatase conjugated goat anti-mouse IgM or IgG, and, finally, a substrate are added. If the primary antibodies have bound to any antigens the secondary antibodies, in turn, bind to them and the substrate reacts

with the enzyme conjugated antibodies and protein bands that have common epitopes are visibly stained. The antibodies can also be characterized on the basis of their binding pattern.

The blocking agent used can qualitatively and quantitatively affect the detection of antigens because of differential binding of antibodies. Wedege and Svenneby (1986) compared four blocking solutions: 0.5% Tween-20 in phosphate buffered saline (PBS), pH 7.2; 0.5% Tween-20 in Tris-saline, pH 10.2; 3% bovine serum albumin (BSA) in PBS, pH 7.2; and 3% BSA in Tris-saline, pH 10.2. They concluded that the antigens detected varied according to the formulation used. BSA, Tween-20 and non-fat dry milk were compared as blocking agents by Spinola and Cannon (1985); they also observed that the binding patterns of antibodies depended upon the blocker used.

Immunoblotting is an excellent procedure that is sensitive and flexible. Nevertheless, complications can arise; therefore, the optimal conditions for transfer and blotting should be formulated for each situation (Towbin and Gordon, 1984).

Simultaneous and consecutive competition assays can be utilized to characterize monoclonal antibodies (Brugemann et al., 1982; Yewdell and Gerhard, 1981). The antibodies can be characterized for their ability to

compete with a labelled antibody, whether the affinity and/or concentration has a greater impact upon the ability of an unlabelled antibody to compete with the labelled antibody, and whether the antibodies compete for one or more binding sites on an antigen (Berzofsky and Berkower, 1984). Antibodies that have been characterized can be utilized in reciprocal competition assays to determine the topographical arrangement of antigenic sites of viruses and other antigens. For example, if the topology of epitopes of several serotypes of CPSPMV and other comovirus was observed, perhaps this could be correlated to the phenotypic expression of virus infectivity in different hosts by the virus serotypes. This information could then be utilized by plant breeders in the development of resistant hybrids and could aid in studies of virus-vector relationships. Biddison et al. (1977) found that a panel of three monoclonal antibodies reacted with each of the influenza virus serotypes Hsw, HO and HI. Therefore, the serotypes were assumed to have antigenic determinants in common. Epitope variability among closely related rabies viruses was observed by Wiktor and Koprowski (1978).

A labelled and an unlabelled antibody are used in competition assays. The labelled antibody can be conjugated with biotin which is detected with an avidin conjugated enzyme and an appropriate substrate. The assay

is read spectrophotometrically. The ability of the unlabelled antibody to compete with the labelled antibody can be monitored by observing a decrease in optical density. Advantages of this system are: (1) biotin has little affect on the binding ability of antibodies when it is bound covalently to them in low molar ratios (Guesdon et al., 1979) and (2) avidin has a very high affinity for biotin (Green, 1963).

Competition can be demonstrated, it is assumed, by performing the following assays: (1) the unlabelled antibody is added prior to the labelled antibody (2) both are added together (Yewdell and Gerhard, 1981). With respect to the former, if the ability of the labelled antibody to bind to the epitopes is decreased by 100%, true competition is likely. When both antibodies are added simultaneously and the binding of the labelled antibody is decreased by 50% or more true competition again is likely. Results obtained from these two assays should corroborate each other.

An antibody that appears to compete with a labelled antibody may not necessarily be doing so. Several other events may occur. (1) The unlabelled antibody may induce conformational changes in the antigen which results in modification of the epitopes. This may preclude binding of the labelled antibody. (2) Epitopes of two antibodies

may overlap or be in close proximity and therefore the labelled antibody cannot bind once the unlabelled one has. (3) Steric hindrance may prevent the second antibody from attaching. Competition assays therefore tend to exaggerate the nearness of epitopes and are most accurate when antibodies do not compete (Yewdell and Gerhard, 1981).

## MATERIALS AND METHODS

## Virus Propagation

CPSMV I, II, III, IV (Lin et al., 1981) and CPMV strain Sb (van Kammen, 1971) viruses were propagated in and purified from Vigna unguiculata Walp. (Blackeye pea, California Blackeye) whereas BPMV was purified from Glycine max cv. Williams (Lin and Hill, 1983). Approximately 0.2 g of infected, desiccated leaf tissue, previously stored at -20 C, was ground in 2 ml of 0.05 M phosphate buffer, pH 7.4 and rubbed manually onto leaves that had been dusted with carborundum (600 mesh). Infected leaf tissue was collected, ground in 0.05 M phosphate buffer, pH 7.4, and once again inoculated onto plants to increase the amount of infected leaf tissue available for purification.

## Purification of Cowpea Severe Mosaic Virus

A modified version of the procedure described by Lin et al. (1981) was used to purify CPSMV.

Systemically infected leaves of cowpeas were harvested approximately 12 days after inoculation and



trituated in 2 volumes (w/v) of borate buffer (0.1 M boric acid, 0.01 M ethylenediamine-tetraacetic acid (EDTA), 0.1% sodium sulfate, pH 7.6) in a Waring blender. Sap was separated from leaf tissue by filtration through cheesecloth. The extract was clarified by adding butanol to a final volume of 8% (v/v). The mixture was incubated at room temperature for 5 minutes and then centrifuged at 11,700 x g for 20 minutes. The supernatant was filtered through glass wool and the virus was precipitated by the addition of polyethylene glycol (PEG; MW 6000) and NaCl to final concentrations of 6% (w/v) and 0.01 M respectively. This solution was incubated at room temperature for 30 minutes followed by a 2-3 hour incubation at 4 C. Precipitated virus was collected by centrifugation at 11,700 x g for 25 minutes. The pellet was resuspended in phosphate buffer (0.05 M  $\text{NaKPO}_4$ , pH 7.0) and placed on a shaker overnight at 4 C.

Further purification was achieved with 2-3 cycles of differential centrifugation. This consisted of centrifuging the resuspended pellet at 8,700 x g for 10 minutes and collecting the supernatant which was then centrifuged at 77,600 x g for 2 hours. The pellet was resuspended in phosphate buffer.

The virus was centrifuged through a sucrose density gradient (10, 20, 30 and 40% sucrose in phosphate buffer)

at 64,700 x g for 1.25 hours in a SW27 swinging bucket rotor (Beckman Instruments, Inc., Palo Alto, CA). An ISCO Density Gradient Fractionator (Instrumentation Specialties Co., Inc., Lincoln, NE, model 640) was used to fractionate the density gradient. CPSMV was detected spectrophotometrically at an absorbance of 254 nm. The virus was dialyzed overnight against phosphate buffer to eliminate the sucrose. Virus was concentrated by centrifuging the dialysate at 77,600 x g for 2 hours. This was resuspended in phosphate buffer and the concentration was determined spectrophotometrically using an extinction coefficient of  $E_{260}^{0.1\%} = 8.0$  (van Kammen, 1971).

#### Purification of Cowpea Mosaic Virus

The purification methods of Herbert (1963) and van Kammen (1967) were modified and used.

Leaves collected from infected cowpeas were frozen overnight and then homogenized in phosphate buffer (0.1 M  $\text{NaKPO}_4$ , pH 7.0; 1 ml/g leaf tissue). The homogenate was filtered through cheesecloth and centrifuged at 10,000 x g for 15 minutes. The virus was precipitated by the addition of PEG (MW 6000) and NaCl to final concentrations of 4% (w/v) and 0.2 M respectively. The mixture was stirred at room temperature for 1 hour and then centrifuged at

10,000 x g for 15 minutes. The resulting pellets were resuspended in phosphate buffer (0.01 M NaKPO<sub>4</sub>, pH 7.0) and placed on a shaker overnight at 4 C. Two differential centrifugation cycles were conducted. The resuspended pellets were first centrifuged at 17,300 x g for 15 minutes; the supernatant was then centrifuged at 77,600 x g for 95 minutes and the pellet was resuspended in phosphate buffer. The virus was placed on a linear sucrose gradient (10, 20, 30 and 40% sucrose in 0.01 M NaKPO<sub>4</sub>, pH 7.0) and centrifuged for 1.25 hours at 64,700 x g in an SW27 rotor. The gradient was fractionated, virus was collected, dialyzed overnight against phosphate buffer and centrifuged at 77,600 x g for 95 minutes. The pellet was resuspended in buffer and the concentration of the virus was determined spectrophotometrically using an extinction coefficient of  $E_{260}^{0.1\%} = 8.1$  (van Kammen, 1967).

#### Purification of Beanpod Mottle Virus

BPMV was purified using a modified version of the protocol of Lin et al. (1983).

Infected plant tissue was harvested and frozen overnight. The frozen tissue was homogenized with phosphate buffer (0.2 M NaKPO<sub>4</sub>, pH 7.2; 2 ml/g leaf tissue) and strained through cheesecloth. The filtrate was

clarified by adding butanol to a final volume of 8% (v/v) and was centrifuged at 11,700 x g for 20 minutes. The supernatant was filtered through glass wool and virus was precipitated by the addition of PEG (MW 6000) to a final concentration of 6% (w/v). The solution was stirred at room temperature until the PEG dissolved, stored at 4 C for 1 to 2 hours and then centrifuged at 11,700 x g for 25 minutes. The pellets were resuspended in phosphate buffer (0.1 M NaKPO<sub>4</sub>, pH 7.2) and put on a shaker overnight at 4 C.

Two cycles of differential centrifugation were done to further purify the virus from contaminating host material. The resuspended pellet was first centrifuged at 8,000 x g for 15 minutes and the supernatant from this was centrifuged at 80,000-100,000 x g for 90 minutes. The pellets were resuspended in 0.1 M NaKPO<sub>4</sub> buffer, pH 7.2.

The virus was further purified as with CPSMV and CPMV on a linear sucrose gradient using a SW27 swinging bucket rotor operating at 64,700 x g for 1.5 hours. The gradient was fractionated, virus was collected, and dialyzed overnight against 0.01 M NaKPO<sub>4</sub> buffer, pH 7.2. The dialysate was concentrated by centrifugation at 62,900 x g for 90 minutes and the concentration determined spectrophotometrically using an extinction coefficient of  $E_{260}^{0.1\%} = 8.7$  (Semancik, 1972).

## Sources of Other Viruses

Maize dwarf mosaic virus strain B (MDMV), barley stripe mosaic virus, strain MI-1 (BSMV), lettuce mosaic virus, strain ATCC PV63 (LMV), soybean mosaic virus, strain Ia 75-16-1 (SMV), and Newcastle's disease virus (NDV) were also used in this study. Purified samples of the the first four viruses were kindly provided by Dr. J. H. Hill and H. Benner whereas NDV was provided by R. Unfer (I.S.U., Ames, IA).

## General Methods

### Distilled and deionized water

Media and buffers were prepared using reagent grade 1 deionized-distilled water (dd H<sub>2</sub>O). This water was produced by removing organic and inorganic ions from distilled water by passing it through a Super-Q Ultra Pure Water System (Millipore Corp., Bedford, MA).

### Production of Monoclonal Antibodies

Monoclonal antibodies were produced using a modification of previously described methods (Van Deusen and

Whetstone, 1981). Each of the monoclonal antibodies will be referred to by their cell line designations. For example, the monoclonal antibody produced by cell line H6 will be called monoclonal antibody H6. Media and reagents are described below.

Dulbecco's modified eagle medium

Dulbecco's modified eagle medium (DMEM) was the nutrient media upon which all of the other media were based. This medium consisted of:

DMEM (Gibco Laboratories, Grand Island, NY, cat. no. 430-2100)	5 L package
N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Sigma Chem. Co., St. Louis, MO, cat. no. H-3375)	35 g
NaHCO <sub>3</sub>	18.5 g
ddH <sub>2</sub> O up to	5 L

The pH of the media was adjusted to 7.2 with 1 N NaOH and it was filtered through a 0.2 um Zetapor filter (AMF Cuno, Meriden, CT, cat. no. NM142-01-020SP) and then through a 0.2 um Acro 50 A filter (Allied Fisher Scientific, Itasca, IL, cat. no. 09-730-241G). Samples were

collected and tested for sterility by incubating the media at 37 C in an atmosphere of 5.1% CO<sub>2</sub>, and at room temperature. The media were stored at 4 C.

#### Growth and maintenance media (DMEM-R)

DMEM-R was used for growth and maintenance of cells, for cloning, and to revitalize cells that had been thawed. It was prepared by adding 1.0 ml of L-glutamine (Sigma, cat. no. G-3126; this was made by adding 2.92 g of L-glutamine to 100 ml of ddH<sub>2</sub>O) and 10 ml of horse-calf serum (2 parts horse to 1 part calf serum; Gibco, cat. nos. 230-6050 and 230-6170 respectively) to 90 ml of DMEM.

#### Myeloma cells

The SP2/0-Ag 14 (SP2/0) cells used were originally supplied by Dr. R.A. Van Deusen (National Veterinary Services Laboratories, Ames, IA). They were maintained by being transferred to DMEM-R tri-weekly.

#### 8-Azaguanine media

When SP2/0 cells are grown, mutants resistant to aminopterin can arise. Eight-azaguanine media was used to

eliminate these mutants (Van Deusen and Whetstone, 1981). This medium was prepared by adding 2 mg of 8-azaguanine (Sigma, cat. no. A-8526) to 1 liter of ddH<sub>2</sub>O and stirring it at 37 C overnight. The procedure for preparing DMEM was then used except 8-azaguanine solution was substituted for ddH<sub>2</sub>O.

#### Conditioned media

Conditioned media was prepared by growing SP2/0 cells in DMEM-R until the media became orangy-yellow. Cells were collected by centrifugation and the media filtered through 0.20 um cellulose acetate filters (Allied Fisher Scientific, cat. no. 09-740-35A) to eliminate remaining cells.

#### HAT media

Hypoxanthine, aminopterin and thymidine (HAT) was used to select hybrids of spleen and SP2/0 cells. When it was added to medium, only fused cells were capable of surviving. Mouse spleen cells that had not fused with SP2/0 cells died within a few days of the fusion. SP2/0 cells also died as they were susceptible to the aminopterin in the HAT medium because they lacked the hypoxan-



thineguanine phosphoribosyltransferase (HGPRT) enzyme. Hybrid cells could survive because the spleen cells express the HPGRT gene and thus the alternative salvage pathway for nucleotide synthesis was utilized (Yelton and Scharff, 1981). HAT medium was prepared in the following manner:

HAT solution (100x)	1 ml
(Hazelton-Dutchland Inc., Denver, CO, cat. no. 59-77076)	
Conditioned media	50 ml
Horse-calf serum	10 ml
DMEM	40 ml
L-glutamine (Sigma, cat. no. G-3126)	1 ml

#### HT media

This medium was prepared in a manner identical to HAT medium except hypothanthine-thymidine (HT; Hazleton-Dutchland Inc., cat. no. 59-57076) was substituted for HAT. HT medium was utilized for the transition of hybridomas from HAT medium to DMEM-R.

### Freezing media

Freezing medium was made by adding 10 ml of dimethyl sulfoxide (DMSO; Sigma, cat. no. D-2650) to 90 ml of horse-calf serum. Hybrids or cells were centrifuged at 225 X g for 8 minutes, resuspended in this medium and stored at -100 C.

### Mouse injection schedule

BALB/c strain mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with 50 ug of CPSMV II emulsified in Freund's complete adjuvant (Sigma, cat. no. F-5881). Twenty-eight and 32 days later an additional 25 ug of virus was injected intravenously. Four days after the second hyperimmunization, the mice were exsanguinated and the serum was kept as a positive control for screening assays.

### Production and maintenance of hybridomas

A modified version of Van Deusen and Whetstone's protocol (1981) for monoclonal antibody production was used. All sterile work was done under a laminar flow hood (Baker Co. Inc., Biddleford, ME).

Following exsanguination, the spleen was removed from the mouse, perfused with approximately 5 ml of sterile DMEM and then separated into a single cell suspension by macerating it through a 100 mesh stainless steel screen. The spleen cells were mixed with SP2/0 cells at a 1:1 ratio and were centrifuged in 50 ml tubes (Corning Glass Works, Corning, N.Y., cat. no. 25330) at 225 X g for 7 minutes. The pellet was resuspended and 1 ml of PEG (MW 1000; Hazleton-Dutchland Inc., cat. no. 59-90739; prepared by solubilizing 2.25 g of PEG in 2.7 ml of PBS) was added over a period of 45 seconds. The cells were mixed gently for 30 seconds and then placed in a 37 C water bath for 90 seconds. One ml of DMEM was added to the cells over a sixty second period and the cells were mixed. After the addition of 10 ml of DMEM, the cells were mixed and incubated for 5 minutes at 37 C. The cells were centrifuged at 225 x g for 8 minutes; the pellet was dispersed with 1 to 2 ml of HAT medium and then resuspended to a concentration of  $5 \times 10^5$  myeloma cells/ml with additional HAT medium. This suspension was plated at 0.2 ml/well in 96 well tissue culture plates (Corning Glass Works, Corning, N.Y., cat. no. 25860), incubated at high humidity, 37 C and 5% CO<sub>2</sub>.

Cells were fed twice with both HAT and HT media and thereafter with DMEM-R respectively, at 2 to 3 day

intervals. When the majority of the wells were from one-half to two-thirds covered with cells they were screened for antibody production by indirect ELISA. Positive cells were cloned by limiting dilution. The procedure used was that of Mernaugh et al. (1987).

The limiting dilution protocol used was as follows. The hybridoma cells growing in wells of tissue culture plates selected by indirect ELISA were resuspended with 2.0 ml serological pipetts (Falcon, Division of Becton, Dickinson and Comp., Oxhard, CA, cat. no. 7507) and 1 drop of the cells was transferred to 5.0 ml of DMEM-R in a sterile Falcon 13 x 100 mm tube (Falcon, Division of Becton, Dickinson and Comp., cat. no. 2027). This was mixed and 1 drop from this suspension was transferred again to 5 ml of DMEM-R. The second cell suspension was mixed and 0.5 ml of it was transferred to 4.5 ml of DMEM-R. The second and third suspensions were plated at 0.2 ml/well into 96 well tissue culture plates. These plates were incubated at high humidity, 37 C and 5% CO<sub>2</sub>. When the majority of the wells were from one-half of two-thirds covered with cells, the wells containing single colonies (which were selected visually using a light microscope) were tested by indirect ELISA for the production of relevant antibodies.

Hybrids that were positive for specific antibody

production were cloned three times. Clones were expanded in DMEM-R and this medium was frozen and used as a source of antibodies. Selected clones were injected into mice to produce ascites fluid.

#### Ascites fluid production

Cloned hybridomas were used to produce ascites fluid. BALB/c mice were primed with 0.5 ml tetramethylpentadecane (Pristane; Sigma, cat. no. T-7640) one to two months prior to being injected intraperitoneally with approximately  $1 \times 10^6$  hybridoma cells suspended in 0.5 ml of PBS. Ascites fluid was collected at two day intervals when the abdominal cavities of the mice became swollen. This normally occurred two to three weeks after the hybridoma cells were injected into the peritoneal cavity. The fluid was clarified by centrifugation at 1000 x g for 8 minutes and was stored at -20 C.

#### Indirect ELISA

The indirect ELISA was employed to screen hybridoma cells during and after the selection process for antibody production. A procedure similar to that described by Voller et al. (1976) was used. The buffers utilized in

this assay are described.

Coating buffer for virus

$\text{Na}_2\text{PO}_4$	7.1 g/ L ddH <sub>2</sub> O
$\text{KPO}_4$	6.8 g/ L ddH <sub>2</sub> O

Sodium phosphate and potassium phosphate were titrated to a pH of 7.0. This buffer was used as a coating buffer for the virus in indirect ELISAs.

Wash buffer (PBS-Tween; 20X)

NaCl	80.0 g
$\text{KH}_2\text{PO}_4$	2.0 g
$\text{Na}_2\text{HPO}_4$	11.9 g
KCl	2.0 g
Polyoxyethylenesorbitan mono- laurate (Tween 20; Sigma, cat. no. P-1379)	5.0 ml
Sodium azide	0.5 g
ddH <sub>2</sub> O to a final volume of	500 ml

The ELISA plates were washed with wash buffer between steps to reduce non-specific binding. Wash buffer was also used to dilute the primary antibody (test fluid) and secondary antibody (alkaline phosphatase conjugated goat

anti-mouse IgM or IgG) and for making the blocking buffer.

Blocking buffer (Blotto)

Non-specific binding was reduced by using blocking buffer.

Antifoam A emulsion	33.3 ul
(Sigma, cat. no. A-5758)	
Non-fat Dry Milk	5 g
(Carnation Brand)	
Wash Buffer to a final volume of	100 ml

Substrate buffer

Substrate buffer was used to prepare the substrate p-nitrophenyl phosphate (Sigma, cat. no. 104-105).

Diethanolamine	10.0 ml
MgCl <sub>2</sub> x 6H <sub>2</sub> O	0.01 g
Sodium azide	0.05 g
ddH <sub>2</sub> O to a final volume of	100 ml

HCl (1 N) was used to adjust the pH to 9.6. The buffer was stored at 4 C.

### Preparation of substrate

One tablet of p-nitrophenyl phosphate (5 mg) was added to 5 ml of substrate buffer.

### Optimization of the indirect ELISA

Several virus concentrations, virus coating buffers and blocking formulations were compared in the indirect ELISA. The most appropriate virus concentration and buffers were determined by calculating the ratio of the absorbance of the positive controls versus the negative controls (P/N ratio) (Hill et al., 1981). The most suitable concentration of CPSMV II was examined because the indirect ELISA had not yet been utilized in any studies involving this virus.

Virus concentrations of 0.5, 1, 2, 4, 5, 7, 9, 15 and 20 ug/ml were compared. Each concentration was applied in duplicate to the wells of an Immulon I ELISA plate which was then incubated at 37 C for 1 hour. Conditioned medium was utilized as the negative control and was used to determine the background reaction. Mouse hyperimmune antiserum was the positive control. The buffers 0.05 M  $\text{NaKPO}_4$ , pH 7.0; 0.05 M  $\text{NaCO}_3$ , pH 9.6, and 0.05 M PBS, pH 7.4, were compared to see which was the optimal coating



buffer for the virus (2 ug/ml). Positive and negative controls were mouse hyperimmune antiserum and conditioned medium, respectively. The blocking buffers that were tested included: horse serum (100%), calf serum (100%), 2% ovalbumin in PBS-Tween, 1% BSA in PBS-Tween, 2% gelatin (Bio-Rad, cat. no. 170-6537) in PBS-Tween, fetal bovine serum (100%) and Blotto. Mouse hyperimmune antiserum was the positive control whereas conditioned media and wells coated with phosphate buffer with no virus were the negative controls. The P/N ratio was calculated from the absorbance values of wells coated with virus versus those with no virus. Indirect ELISAs were performed as described below and contents of the wells were quantified spectrophotometrically.

#### Indirect ELISA assay

CPSMV II (2 ug/ml; 50 ul/well) in coating buffer was added to polystyrene Immulon I plates (Dynatech Laboratories, Alexandria VA.). The plates were incubated for 1 hour at 37 C and washed 3 times with wash buffer for 3-5 minutes. They were then blocked with 300 ul of blocking buffer for either 1 hour at 37 C or overnight at 4 C and washed again. Fifty microliters of test fluid from the hybridoma cultures, hyperimmune serum, or conditioned

medium were added to each well and plates were incubated for 1 hour at 37 C. The plates were washed 3 times as above to remove unbound proteins. Alkaline phosphatase conjugated goat anti-mouse IgM or IgG antibodies (50 ul/well diluted 1:1000 in PBS-Tween; Sigma, cat. nos. A-7784 and A-1902 respectively) were added and incubated for 1 hour at 37 C. The plates were washed and 50 ul/well of substrate was added. They were subsequently incubated for 30 to 60 minutes at 37 C. Reactions were stopped by adding 50 ul of 3 N NaOH to each well. The reaction could be observed visually or the absorbance measured at 410 nm with a Dynatech Minireader II (Dynatech Laboratories). Conditioned media was used as a negative control since it contained no antibodies and hyperimmune serum was the positive control.

#### Determination of Immunoglobulin Class and Subclass

The class and subclass of monoclonal antibodies were determined using a modification of the indirect ELISA described above.

Virus, blocker and monoclonal antibodies in DMEM-R medium were applied to ELISA plates (50 ul/well). Fifty microliters/well of rabbit anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA (Zymed Laboratories Inc., San Fran-

cisco, CA, cat. no. 90-6550) were added to the microtiter plates. The plates were incubated for 1 hour at 37 C and washed 3 times with wash buffer. Peroxidase-labelled affinity purified goat anti-rabbit IgG (H and L) antibody (Zymed Lab., cat. no. 90-6550) was added (50 ul/well). Plates were incubated for 1 hour at 37 C and washed 3 times. One-hundred microliters of substrate was added to all wells, the plates were incubated at room temperature for 30-60 minutes and contents of the wells were read visually or with a spectrophotometer at 410 nm. The substrate used was made by combining 5 ml of 0.05 M citric acid, pH 4.0, with 20 ul of 3% H<sub>2</sub>O<sub>2</sub> and 25 ul of 40 mM ABTS solution (2, 2, azino-di-(3-ethylbenzthiazoline sulfonic acid)).

#### Purification of Monoclonal Antibodies from DMEM-R Media

IgM was purified from DMEM-R media on an anti-mouse IgM (u-chain specific) agarose column (Sigma, cat. no. 4540). The protocol used was as follows (Thomas D. Hillson, I.S.U., Ames, IA; personal communications).

Approximately 250 mls of DMEM-R was applied to a 5 ml column bed of agarose. The column was washed with 4 bed volumes of 0.01 M PBS, pH 7.4, to eliminate contaminating

compounds. The IgM was eluted by adding 10 ml each of 0.01 M PBS at pH 6.0, 4.0 and 3.0, respectively. The eluted antibodies were titrated with 0.01 M dibasic PBS to a pH of 7.5. Fractions were tested by indirect ELISA, pooled and dialyzed overnight against 0.01 M PBS, pH 7.4. Aliquots were stored at -20 C.

The Antigenic Analysis of Comoviruses and  
Other Antigens, and the Characterization  
of Monoclonal and Polyclonal Antibodies  
by Immunoblotting

Immunoblot assays were utilized to determine the antigenic relationships of ten plant and one animal virus and, several other antigens, and concurrently, to characterize monoclonal and polyclonal antibodies by comparing which antigens they bind to (their binding patterns).

Viruses, other antigens and antibodies utilized in immunoblotting

The viruses and other antigens utilized in this study are presented in Table 3. Monoclonal antibodies produced against CPSMV I, II, MDMV (MAGII-IgG and MBMIII-IgM), BSMV (B2-IgG and B5-IgM), SMV (S1 and S4; both IgG), NDV (IgG)

and the idiotype monoclonal antibody produced against SMV (anti-S1; IgG) were utilized for determining which of the antigens were related. The antibodies were kindly provided by F. E. Jones (MDMV), T. Permar (BSMV and SMV), R. Unfer (NDV) and R. Merbaugh (anti-S1) all produced at I.S.U., Ames, IA. Mouse hyperimmune antiserum produced to CPSMV II and peritoneal fluids from an apparently healthy mouse primed with pristane were also used as positive and negative controls, respectively.

#### Disruption of viral proteins

Viral antigens were dialyzed against 0.125 M Tris-HCl containing 0.1% mercaptoethanol (2-MCE), pH 7.6. Antigens (1 mg/ml) were disrupted by adding an equal volume of 0.125 M Tris-HCl containing 1% sodium dodecyl sulfate (SDS), 1% 2-MCE, pH 6.8, followed by heating at 100 C for 17 minutes except CPMV which was heated at 95 C for 5 minutes. The antigens were then chilled in an ice bath for 2-5 minutes. CPMV was handled differently because the incubation conditions used can affect virus disruption and electrophoretic resolution of the capsid protein into 2-3 or 5-6 bands according to Geelen et al. (1972) who further reported that a 5 minute incubation at 95 minutes resulted in 2-3 bands being resolved electrophoretically. This

number of bands was desired for the assay since this virus is composed of 2 proteins; therefore, a 5 minute incubation at 95 C was utilized.

### SDS polyacrylamide gel electrophoresis

Large biological molecules can be separated by gel electrophoresis on the basis of charge and size. The pH of a gel greatly affects the charge of a protein which in turn influences its mobility. The mobility of a molecule also depends upon the pore size of a gel. Two systems can be used in gel electrophoresis generally referred to as the continuous and discontinuous systems. In the continuous system, the same buffer is used throughout the gel and therefore only one layer of gel is necessary. The discontinuous system utilizes at least 2 layers of gel, the stacking and resolving gel, with different pH and pore size, respectively (Takacs, 1979).

The stacking is the first gel through which protein passes and it contains an ion (leading ion) whose electrophoretic mobility is greater than that of the protein. The gel is placed in a tank with a buffer which contains an ion of less mobility (trailing ion) than the protein. During electrophoresis the protein is sandwiched between the leading and trailing ions and is stacked at

the boundary between the stacking and resolving gels. Below the stacking gel is the resolving gel. The pore size of this gel is smaller than the stacking gel and the pH is higher. The mobility of the trailing ion increases as it passes into the resolving gel; it moves ahead of the protein so that the latter separates on the basis of size and charge (Takacs, 1979).

The gels and solutions used in this system are as follows:

Acrylamide solution

This solution was used to make stacking and resolving gels.

Acrylamide (Bio-Rad, cat. no. 161-0101)	24 g
Bis-acrylamide (Bio-Rad, cat. no. 161-0201)	0.64 g
ddH <sub>2</sub> O to	90 ml

Stacking gel buffer, pH 6.8

This buffer was used for preparation of the stacking gel.

Tris (Sigma, cat. no. T-1378)	1.84 g
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Sodium dodecyl sulfate (SDS)	0.12 g
(Bio-Rad, cat. no. 161-0302)	
ddH <sub>2</sub> O to	100 ml

The pH was adjusted to 6.8 with 1 N HCl.

### 5% Stacking gel

The stacking gel was prepared as follows.

Acrylamide solution	830 ul
Stacking gel buffer	4.11 ml
10 % Ammonium persulfate	30 ul
(0.1g in 0.9 ml ddH <sub>2</sub> O;	
Bio-Rad, cat. no. 161-0700)	
N, N, N, N-Tetramethylethylenedi-	30 ul
amine (Temed; Bio-Rad, cat. no.	
161-0801)	

### Resolving gel buffer, pH 8.8

This buffer was used in the preparation of the resolving gel.

Tris	18.33 g
SDS	0.045 g
disodium EDTA	0.118 g
ddH <sub>2</sub> O	100 ml



The pH was adjusted to pH 8.8 with 1 N NaOH.

15% resolving gel

The resolving gel was prepared as follows.

Acrylamide solution	20.0 ml
Resolving gel buffer	9.9 ml
ddH <sub>2</sub> O	9.9 ml
10% Ammonium persulfate	80 ul
Temed	80 ul

Coomassie blue stain

Coomassie blue stain was used to stain and fix the gel.

Coomassie brilliant blue (Bio-Rad, cat. no. 161-0400)	0.5 g
Methanol	228 ml
ddH <sub>2</sub> O	228 ml
Glacial acetic acid	45 ml

Destain solution

Polyacrylamide gels were destained so that the protein bands could be visualized.

Methanol	350 ml
Glacial acetic acid	100 ml
ddH <sub>2</sub> O to	1750 ml

### Gel storage

Gels were stored in a solution containing 7% glacial acetic acid in ddH<sub>2</sub>O.

### Electrode buffer, pH 8.3

Electrode buffer contains ions that are involved in resolving protein bands during electrophoresis. This buffer consisted of the following.

Tris	3.0 g
Glycine (Sigma, cat. no. G-7126)	14.4 g
SDS	1.0 g
Disodium EDTA	0.292 g
ddH <sub>2</sub> O to	1000 ml

The pH was adjusted to pH 8.3 with 1 N NaOH.

### Sample buffer (tracking dye), pH 6.8

Tracking dye was mixed with virus samples so that the progress of electrophoresis could be estimated. It con-

sisted of:

Tris	9.8 g
SDS	10.0 g
2-mercaptoethanol	20 ml
Glycerol	150 ml
Bromophenol blue	0.02 g
(Bio-Rad, cat. no. 161-0404)	
ddH <sub>2</sub> O to	1000 ml

The pH was adjusted to pH 6.8 with 1 N HCl.

Disrupted antigens were electrophoresed on the discontinuous polyacrylamide gel system described by Laemmli (1970). Thirteen microliters of sample buffer was mixed with an equal volume of antigen (1 mg/ml) for each well on the polyacrylamide gel. Once all of the wells were filled, the gel was electrophoresed at 75 volts using a Protean Slab Gel Electrophoresis Instrument (Bio-Rad, Protean Dual 16 cm Slab Cell, 16 cm X 18 cm X 1.5 mm, cat. no. 165-1420) until the tracking dye had passed into the resolving gel. At that time, the voltage was increased to approximately 100 volts and electrophoresis continued until the tracking dye reached the bottom of the gel.

Immunoblotting procedure

Proteins were electroeluted from polyacrylamide gel to nitrocellulose according to methods described by Gershoni and Palade (1983), and Towbin and Gordon (1984). The buffers used in immunoblotting include the following:

Transfer buffer

Transfer buffer was used when electroeluting proteins from polyacrylamide gel to nitrocellulose. The buffer used contained methanol which stabilized the gel during transfer and increased the capacity of nitrocellulose to bind proteins (Gershoni and Palade, 1983). This buffer contained the following:

Tris	9.08 g
Glycine	43.3 g
ddH <sub>2</sub> to	3000 ml
Adjust pH to 8.3 with 1 N NaOH.	
Methanol	750 ml

TBS-Tween, pH 7.4

The nitrocellulose was rinsed with TBS-Tween between each step to reduce non-specific binding. This buffer was

also utilized to dilute alkaline phosphatase conjugated goat anti-mouse IgM or IgG. The buffer consisted of:

Tris	18.15 g
NaCl	25.5 g
Tween 20 (Sigma, cat. no. P-1379)	1.5 ml
ddH <sub>2</sub> O to	3000 ml

The pH was adjusted to 7.4 with 1 N NaOH.

#### Blocking buffer

The nitrocellulose was blocked following the transfer to reduce non-specific binding. The buffer consisted of 3% gelatin in TBS-Tween.

#### Primary antibody buffer

The monoclonal antibodies (primary antibodies) were diluted in TBS-Tween containing 1% gelatin.

#### AP 7.5 buffer

Nitrocellulose was rinsed 3 times with AP 7.5 to activate it. The buffer consisted of:

Tris	12.11 g
NaCl	5.84 g

MgCl x 6H<sub>2</sub>O 0.41 g

Triton X-100 (Sigma, cat. no. T-6878) 0.5 ml

The pH was adjusted to 7.5 with 1 N NaOH.

#### AP 9.5 buffer

AP 9.5 was used to wash the nitrocellulose and to prepare it for the enzyme-substrate reaction. The buffer consisted of:

Tris 12.11 g

NaCl 5.84 g

MgCl<sub>2</sub> 1.02 g

The pH was adjusted to 9.5 with 1 N NaOH.

#### NBT-BCIP

Nitro blue tetrazolium (NBT; Sigma, cat. no. N-6876) when mixed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, cat. no. B-8503) is a sensitive substrate that reacts with alkaline phosphatase.

A 15 ml volume of NBT-BCIP was made by suspending 5 mg of NBT in 1.5 ml of AP 9.5 buffer in a 1.5 ml micro-centrifuge tube (USA/Scientific Plastics Inc., Ocala, FL, cat. no. USA-505) which was vortexed for 1-2 minutes. A pellet was collected by centrifugation for 30 seconds.

The supernatant was decanted into 10 ml of warm AP 9.5 buffer (35 C). The pellet was extracted an additional 2 times. BCIP (2.5 mg) was dissolved in 50 ul of N,N-dimethyl formamide and slowly added to the NBT solution. Substrate was stored at 35 C and used the day it was prepared (Leary et al., 1983).

Color stop, pH 7.5

This reagent was utilized to stop the enzyme-substrate reaction and as a storage buffer for the nitrocellulose. It consisted of:

Tris	1.21 g
Disodium EDTA	0.29 g
ddH <sub>2</sub> O	1000 ml

The pH was adjusted to 7.5 with 1 N NaOH.

After the proteins were electrophoresed on polyacrylamide gel, they were transferred from the gel to nitrocellulose (Millipore Corp., Bedford, MA, cat. no. HAHY 000 10) using a procedure similar to that described by Towbin et al. (1979). A sponge pad (Bio-Rad, cat. no. 170-3908) which had been soaking in transfer buffer was placed on top of a perforated plastic support (Bio-Rad, cat. no. 170-3907). Whatman 3MM filter paper, the gel, a

second piece of filter paper, sponge pad and finally plastic support were added. This assembly was placed in a transfer chamber (Bio-Rad Trans-Blot Cell, cat. no. 170-3905) with the nitrocellulose facing towards the anode. The transfer was made at 75 volts for 3 hours and then the nitrocellulose was removed from the transfer system.

Proteins that had been transferred were detected using immunological techniques (Gershoni and Palade, 1983). The nitrocellulose was washed 3 times with TBS-Tween, placed in a seal-a-meal bag (Dazey Corporation, Industrial Airport, KS; Dazey Micro-Seal, Model 6008) and blocked for 30 minutes at 37 C on a rotator (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ, Adams Nutator, model no. 1105). The nitrocellulose was then washed 3 times as above and incubated at room temperature overnight with the primary antibody. The next day the nitrocellulose was washed 3 times with TBS-Tween and alkaline phosphatase conjugated goat anti-mouse IgM or IgG was added (diluted 1/1000 in TBS-Tween). This was incubated for 1 hour at 37 C on the rotator. The nitrocellulose was washed 3 times with TBS-Tween and placed on a tray. AP 7.5 and AP 9.5 buffers were added 3 times and 2 times respectively for 2 minutes, to rinse and activate the nitrocellulose. It was then incubated at room temperature with NBT-BCIP for 1 or 2 hours and then



color stop was added. The nitrocellulose was stored in color stop in the dark.

#### Modifications of immunoblot assays

Four blocking buffers were compared to see whether the binding of antibodies and, therefore, the detection of antigens, was affected. They included: Blotto; 3% BSA in 0.01 M PBS, pH 7.5; 3% BSA in 0.02 M Tris-0.15 M saline, pH 10.2; and 50% fetal bovine serum in PBS-Tween. Monoclonal antibody 2-7A (Table 2) was also diluted in PBS-Tween with 1% BSA or 1% gelatin to see whether this had an impact on its binding pattern. This antibody was selected because it had a high titer as determined by indirect ELISA. Assays were also conducted to determine if alkaline phosphatase goat anti-mouse IgM from Sigma (Sigma, cat. no. A-7784) and from Cappel (Cappel Worthington Biochemical, Malvern, PA, cat. no. 8611-0201), both diluted 1/1000, or conditioned media reacted non-specifically with several antigens.

#### Serologically Specific Electron Microscopy

Electron microscopy was utilized to determine whether CPSMV II and SMV were related serologically. IgM purified

from cell line 4-5B (Table 2) was used and observations were made whether this antibody bound specifically to CPSMV II and/or SMV. The protocol utilized was as follows. Virus was added to a copper grid and the grid was incubated for 10 minutes at room temperature. The grid was washed with ddH<sub>2</sub>O, the purified IgM was added, and the grid was incubated at room temperature for 1 hour. The grid was washed with ddH<sub>2</sub>O and gold labelled goat anti-mouse IgM (Boehringer Mannheim Biochemicals by Janssen Pharmaceuticals, Beerse, Belgium, cat. no. 605-750) was added. This was used as the detection antibody. The assay was performed by R. Mernaugh of our laboratory.

#### Characterization of Monoclonal Antibodies by Competition Assays

Competition assays were utilized to characterize 17 monoclonal antibodies. The antibodies were characterized for their ability to compete with a second labelled antibody, the effect that their affinities or concentrations had upon their ability to compete, and whether the antibodies competed for one or two binding sites on the CPSMV I antigen. The assays were conducted using biotinylated antibodies.

### Biotinylation of antibodies

The purified antibodies were biotinylated by the method of Bayer et al. (1979). N-Hydroxysuccinimido-biotin (BNHS; 1.25 mg; Sigma, cat. no. H-1759) was dissolved in 0.5 ml of dimethylformamide (DMF). This was added to the antibody preparation produced by cell line H6 (Table 2) at a 1:50, v/v, 50:1, mol/mol ratio, rotated for 4 hours at room temperature, stored at 4 C overnight, and dialyzed against 0.01 M PBS, pH 8.0, for 24 hours at 4 C.

### Competition assay procedure

The method described earlier for the indirect ELISA was further modified for use in the competition assay. The biotinylated antibody H6 used in the assay was developed against serotype I of CPSMV (this monoclonal antibody was made using procedures similar to those previously described and was kindly provided by Dr. J.H. Hill, I.S.U., Ames, IA); thus, CPSMV I (4 ug/ml; 50 ul/ml) was used to coat the ELISA plates. Monoclonal antibody H6 was selected as the the labelled antibody because it had the highest titer as determined in an indirect ELISA. Biotinylated antibody H6 (2.5 ug/ml; 50 ul/well) was added to two wells on each plate. This was considered the

positive control because no competing antibodies were included; therefore, no competition could take place resulting in a maximum binding of the biotinylated antibody. Moreover, these wells were designated as having an absorbance of 100% and were used as a basis for comparison to quantitate the competition of each individual monoclonal antibody. This was accomplished by measuring the amount that the competing antibody decreased the maximum (100%) absorbance due to antibody H6. SMV was also added to two wells/plate as one of the negative controls since the biotinylated antibody did not react with SMV. The second negative control was 0.01 M  $\text{NaKPO}_4$  buffer, pH 7.0, with no virus (two wells/plate); it was used to assess whether the biotinylated antibody reacted non-specifically with the blocking agent.

The concentrations of the biotinylated antibody H6, virus and avidin conjugated alkaline phosphatase required, were optimized in the following manner. Virus concentrations of 8, 6, 4, 2, 1, 0.5, 0.25, 0.125  $\mu\text{g/ml}$  in 0.05 M  $\text{NaKPO}_4$  buffer, pH 7.0 (50  $\mu\text{l/well}$ ), were added to ELISA plates which were then incubated at 37 C for 1 hour. Plates were washed with PBS-Tween and Blotto was added (400  $\mu\text{l/well}$ ). The plates were incubated overnight at 4 C and then washed. Biotinylated antibody concentrations of 5, 2.5, 1.25, 0.625, and 0.312  $\mu\text{g/ml}$  (50  $\mu\text{l/well}$ ) in PBS-

Tween were applied and plates were incubated for 90 minutes at 37 C. The plates were washed and 50 ul/well of avidin-conjugated alkaline phosphatase (Sigma, cat. no. A-2527) at concentrations of 8, 3, 1 and 0.25 ug/ml were added and plates were incubated at 37 C for 90 minutes. The plates were washed and substrate (50 ul/well) was applied. The absorbance was read on a Dynatech microplate reader (Dynatech Laboratories) after plates were incubated for 1 and 2 hours at 37 C.

After the concentrations of virus, biotinylated antibody and avidin conjugated alkaline-phosphatase were optimized, competition assays were performed. Seventeen monoclonal antibodies were serially diluted from 1/1 (undiluted) to 1/1024 or 1/100,000, depending upon the antibody, in PBS-Tween. Two different competition assays, the simultaneous and consecutive assays, were performed to determine which of the antibodies competed with the biotinylated antibody. The assessment of whether an antibody did or did not compete was ascertained in the following manner. The assumption was made that an unlabelled antibody did not compete with the biotinylated antibody if in both the consecutive and simultaneous assays the absorbance of an unlabelled antibody was greater than 75% at all dilutions (1/1 (undiluted) to 1/1024 or 1/100,000), whereas absorbance values below 74%

indicated competition. Furthermore, greater competition in the simultaneous assay indicated the concentration of the unlabelled antibody had the greater affect on its ability to compete while greater competition in the consecutive assay indicated the affinity had a greater impact. These two assays were also utilized to suggest the number of binding sites that the unlabelled antibodies reacted with.

The protocols of the consecutive and simultaneous assays are described below. In the consecutive assay, serial dilutions of the primary antibody, ranging from 1/1 (undiluted) to 1/1024 or 1/100,000 were added to the ELISA plates without the biotinylated antibody H6. The plates were incubated at 37 C for 90 minutes and washed. Biotinylated antibody H6 (2.5 ug/ml; 50 ul/well) was applied, plates were incubated for 90 minutes at 37 C and washed. Avidin conjugated alkaline phosphatase (3 ug/ml; 50 ul/well) was added, the plates were incubated for 90 minutes and then washed. Substrate was applied (50 ul/well), the plates were incubated at 37 C and were read spectrophotometrically after 1 and 2 hours. The simultaneous assay involved adding serial dilutions of the unlabelled antibodies (50 ul/well), ranging from 1/1 to 1/1024 or 1/100,000, simultaneously with the biotinylated antibody H6 (2.5 ug/ml; 50 ul/well). The plates were

incubated for 1 hour at 37 C and washed. Avidin conjugated alkaline phosphatase (3 ug/ml; 50/well) was applied; plates were incubated at 37 C for 90 minutes and washed. Substrate was added (50 ul/well) and plates were read spectrophotometrically at an absorbance of 410 nm after 1 and 2 hour incubations at 37 C.

## RESULTS

## Production of Monoclonal Antibodies

Hybridoma cells were pipetted into 1308 wells of tissue culture plates. Of these, 929 or 71% of the wells contained fused cells; 27% or 251 of these cells produced antibodies reactive to CPSMV II. Forty-five antibody producing hybrids were cloned by limiting dilution; however, only 11 or 24% of the 45 hybrids continued to produce antibodies after being cloned approximately three times. These 11 cell lines were expanded in DMEM-R medium to produce antibodies. Ascites fluid was produced from the cell lines 4-5B, B12, 2-11A, and 1-11G (Table 2). Each culture was stored at -90 C or with 0.1% sodium azide at 4 C. Six cell lines, developed against CPSMV I using similar procedures, were kindly provided by Dr. J. H. Hill (I.S.U., Ames, IA).

## Optimization of Indirect ELISA

Several virus (CPSMV II) concentrations, virus coating buffers and blocking formulations were compared in the indirect ELISA. The most appropriate concentration of



virus appeared to be 2 ug/ml since at this concentration the ELISA was sensitive but had low background readings, i.e., it had the highest P/N ratio (Fig. 1). The average optical densities of the reaction involving the coating buffers for CPSMV II (2 ug/ml) were 2.05, 2.01 and 1.98 for  $\text{NaKPO}_4$ ,  $\text{NaCO}_3$  and PBS respectively. These results indicate that all the buffers were comparable and could be used equally well in the indirect ELISA. Several blocking formulations were tested. Blotto was the optimal agent since it was an effective blocking agent and resulted in low background readings. Gelatin and BSA were not quite as effective but were acceptable since their P/N ratio was 7.65 and 7.56, respectively; however, the remainder were not acceptable since they had very low P/N ratios (Table 1).

#### Isotyping of Monoclonal Antibodies

The class of monoclonal antibodies produced by each cell line was determined using the isotyping kit from Zymed Laboratories. The results are shown in Table 2. Sixteen of the hybrids produced IgM and one produced IgG2a.

### Purification of Antibodies

Monoclonal antibodies produced by cell line H6 were purified from DMEM-R medium on an anti-mouse IgM (u chain specific) agarose column. They were eluted from the column when PBS buffer, pH 6.0 was added and collected as one ml fractions. The absorbance of each of the fractions was determined at 280 nm and the immunological activity was assessed by indirect ELISA using CPSMV I (2 ug/ml). The concentration of the purified antibodies was calculated. Approximately 4.76 mg of IgM was purified from 250 ml of DMEM-R medium containing monoclonal antibody H6.

### Immunoblot Analysis

Immunoblot analyses were utilized to determine the antigenic relationships of 10 plant viruses, one animal virus and several other antigens, and to characterize monoclonal and polyclonal antibodies.

CPSMV I, II, III, IV, BPMV and CPMV when disrupted and resolved on polyacrylamide gel yielded three bands. When immunoblot assays were conducted, all of these bands reacted with most of the monoclonal and polyclonal antibodies that they were probed with (Fig. 2 and Table 4).

This was true whether IgM or IgG antibodies were used. The other viruses, when disrupted, were separated on the gel into one to eight bands, some of which cross-reacted with antibodies (Table 4 and Fig. 3). For example, none of the antibodies tested against SMV reacted with any of the SMV bands, approximately one-half of the antibodies tested against MDMV and BSMV reacted with a number of the MDMV and BSMV proteins, and all of the antibodies tested against LMV reacted with LMV bands (Fig. 3). Monoclonal antibody 4-5B reacted with plant proteins from uninoculated cowpeas (Fig. 4). The anti-idiotypic antibody (anti-S1) did not react with CPSMV II, LMV or S1. Hyperimmune serum and peritoneal fluid from a mouse primed with pristane reacted with CPSMV II but not MDMV. The peritoneal fluid also reacted with BPMV. Conditioned media did not react with CPSMV II but did with the molecular weight standards. All of the antibodies tested reacted with the molecular weight standards phosphorylase B, ovalbumin, soybean trypsin inhibitor and lysozyme (Fig. 5).

Four different blocking formulations were compared to see whether they modified the binding of monoclonal antibody 4-5B and therefore the detection of antigens by this antibody (Table 5). The blocking agents tested included: Blotto, 3% BSA in 0.01 M PBS-Tween, pH 7.5, 3% BSA in 0.02 M Tris-0.15 M saline, pH 10.2, and 50% fetal

bovine serum in PBS-Tween. The binding of antibody 4-5B with CPSMV II and the molecular weight standards was not affected by the formulation used. The fetal bovine serum formulation was used in additional assays involving the monoclonal antibody 4-5B and the antigens BPMV, CPMV, BSMV, and LMV. This antibody did not bind with MDMV and SMV but did with BPMV and BSMV. The pattern of binding observed in the assays mentioned above was identical to what was originally observed when the standard blocking agent 3% gelatin in TBS-Tween was utilized. Therefore the blocking agents had no impact upon the binding of the antibody 4-5B and the detection of the antigens. Antibodies from clone 2-7A were diluted in PBS-Tween with 1% BSA and PBS-Tween with 1% gelatin (Table 5). In both situations the antibody did react with CPSMV II and thus this modification had no impact upon binding. Alkaline phosphatase conjugated goat anti-mouse IgM from two commercial sources (Sigma and Cappel) did not react with CPSMV II (Table 4); however, the alkaline phosphatase conjugate from Cappel did react with the molecular weight standards (Table 4).

### Electron Microscopy Studies

Electron microscopy was used to determine whether CPSPV II and SMV were related serologically. IgM purified from cell line 4-5B (Table 2) bound with CPSPV II but not with SMV (Figs. 6 and 7). This agrees with results obtained by immunoblot analysis where purified IgM from cell line 4-5B reacted with CPSPV II but not with SMV (Table 4).

### Competition Assays

The optimum concentration of the virus, biotinylated monoclonal antibody H6, and avidin conjugated alkaline phosphatase for the competition assay were determined to be 4, 2.5 and 3 ug/ml, respectively.

Monoclonal antibodies were characterized in the following manner. The dilution profiles of the consecutive and simultaneous assays were studied in order to determine which of 17 unlabelled monoclonal antibodies competed with the biotinylated antibody H6, whether the affinity or the concentration of the antibodies had a greater impact upon their ability to compete, and whether the antibodies competed for one or two sites on CPSPV I. Antibodies that did not compete included 2-11G, G10, 1-9G,

4-6F and 1-11G (Fig. 8 M-Q). The remaining antibodies did compete; namely, 4-5B, 3-9C, 5B, 4-5F, 2-1F, B12, 6A, 3-11C, 2-7A, 2-11A, F1, and H6 (Fig. 8 A-L). A summary of the competing and non-competing antibodies is shown in Table 6.

Figure 1. Determination of the most appropriate virus concentration (CPSMV II) for indirect ELISA. This was determined by calculating the P/N ratio (○—○) which was the ratio of the absorbance of the positive control (hyperimmune antiserum; ◆—◆) versus the negative control (conditioned medium; □—□)

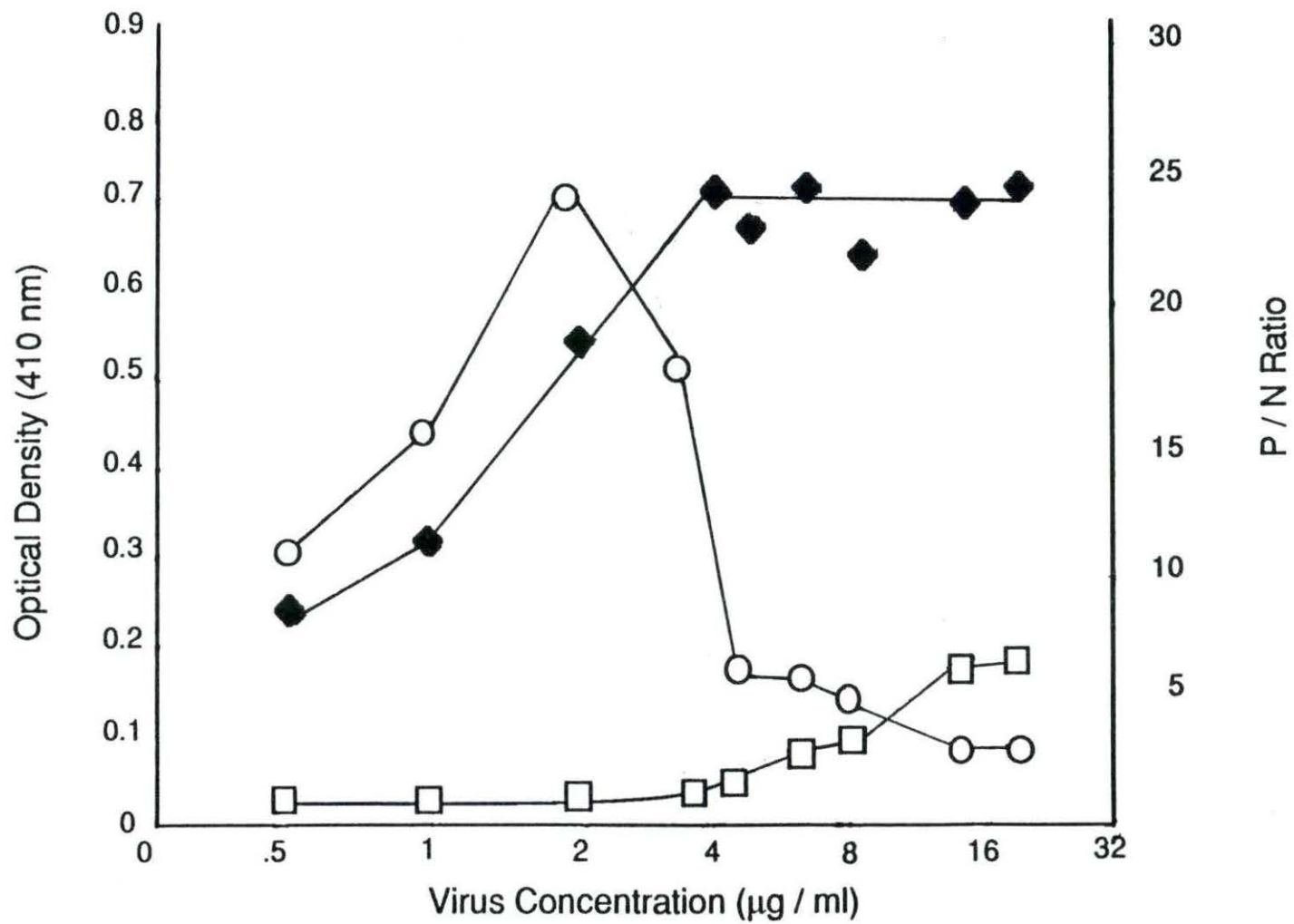




Table 1. Comparison of blocking buffers in the indirect ELISA for the detection of CPSMV II

Blocking formulation	Absorbance, virus <sup>a</sup>	Absorbance, no virus <sup>b</sup>	P/N Ratios <sup>c</sup>
Fetal bovine serum (100%)	1.85	1.29	1.43
2% gelatin in PBS-Tween	1.76	0.23	7.65
1% BSA in PBS-Tween	1.89	0.25	7.56
2% Ovalbumin in PBS-Tween	1.99	1.19	1.67
Blotto	1.79	0.06	29.83
Horse serum (100%)	1.73	1.02	1.70
Calf serum (100%)	1.83	1.10	1.66

<sup>a</sup>Absorbance at 410 nm of CPSMV II in 0.05 M NaKPO<sub>4</sub> buffer, pH 7.0, at a concentration of 2 ug/ml (P).

<sup>b</sup>Absorbance at 410 nm of 0.05 M NaKPO<sub>4</sub> buffer, pH 7.0, with no CPSMV II (N).

<sup>c</sup>The P/N ratio was calculated as the ratio of the absorbance of the positive and negative controls; these were the wells coated with CPSMV II in NaKPO<sub>4</sub> buffer versus those coated with NaKPO<sub>4</sub> buffer with no virus, respectively. Hyperimmune antiserum (1/5 dilution) was used as the test antibody.

Table 2. Isotype of monoclonal antibodies developed against CPSMV serotypes I and II as determined by indirect ELISA<sup>a</sup>

Monoclonal antibody	Class	Subclass	Source
1-9G	IgM	----- <sup>b</sup>	CK <sup>c</sup>
1-11G	IgM	-----	CK
2-1F	IgM	-----	CK
2-7A	IgM	-----	CK
2-11A	IgM	-----	CK
2-11G	IgM	-----	CK
3-9C	IgM	-----	CK
3-11C	IgM	-----	CK
4-5B	IgM	-----	CK
4-5F	IgM	-----	CK
4-6F	IgM	-----	CK
B12	IgG	2a <sup>d</sup>	JH <sup>e</sup>
5B	IgM	-----	JH
G10	IgM	-----	JH
F1	IgM	-----	JH
6A	IgM	-----	JH
H6	IgM	-----	JH

<sup>a</sup>CPSMV I and II concentrations of 2 ug/ml were used in the indirect ELISA.

<sup>b</sup>Not applicable to this antibody.

<sup>c</sup>Monoclonal antibodies developed by C. Kubanek using the immunogen CPSMV II.

<sup>d</sup>Subclass of IgG antibody.

<sup>e</sup>Monoclonal antibodies developed by Dr. J. H. Hill using the immunogen CPSMV I.

Table 3. Viruses and other antigens utilized in immunoblot assays

Virus or antigen	Source
CPSMV I, II, III and IV	C.K. <sup>a</sup>
CPMV strain Sb	C.K.
BPMV	C.K.
MDMV strain B	J.H. and H.B. <sup>b</sup>
BSMV strain MI-1	J.H. and H.B.
LMV strain ATCC PV63	J.H. and H.B.
SMV strain Ia-75-16-1	J.H. and H.B.
NDV	R.U. <sup>c</sup>
Idiotypic antibody S1 <sup>d</sup>	E.H. <sup>e</sup>
Molecular weight standards <sup>f</sup>	Bio-Rad
Cowpea plant sap <sup>g</sup>	C.K.

<sup>a</sup>Viruses provided by C. Kubanek.

<sup>b</sup>Viruses provided by Dr. J. H. Hill and H. Benner.

<sup>c</sup>Viruses provided by Robert Unfer.

<sup>d</sup>Monoclonal antibody S1 produced against SMV.

<sup>e</sup>S1 antibody was produced and supplied by E. Hill (Hill et al., 1984).

<sup>f</sup>Molecular weight standards (lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B; Bio-Rad, cat. no. 161-0304).

<sup>g</sup>Plant sap from uninfected cowpeas cv. Calif. Blackeye supplied by C. Kubanek.

Figure 2. Immunoblot analysis of CPSMV I, II, III, CPMV, CPSMV IV, and BPMV in lanes 1, 2, 3, 4, 5 and 6, respectively. Viral proteins were disrupted by adding an equal volume of 0.125 M Tris-HCl, pH 6.8, containing 1% sodium dodecyl (SDS) and 2-mercaptoethanol (2-MCE), followed by heating at 100 C for 17 minutes except CPMV which was heated at 95 C for 5 minutes. Proteins were resolved electrophoretically, transferred to nitrocellulose and probed with monoclonal antibody 1-11G. This figure is representative of the binding pattern of other monoclonal and polyclonal antibodies that were tested against and reacted with these viruses (see Table 5). The molecular weights of the large and small proteins are 39-50 K and 20-25 K, respectively

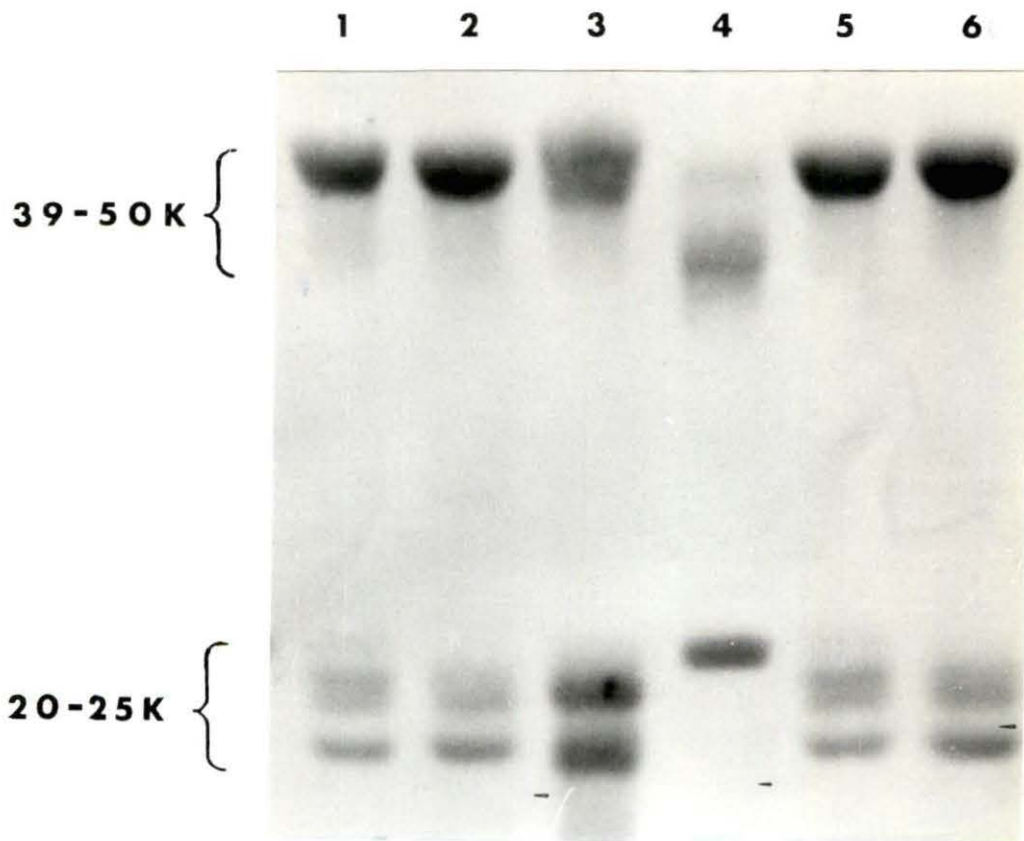


Figure 3. Immunoblot analysis of BSMV, LMV and MDMV in lanes 1, 2 and 3, respectively. Viral proteins were disrupted by adding an equal volume of 0.125 M Tris-HCl containing 1% 2-mercaptoethanol (2-MCE) and sodium dodecyl sulfate (SDS), pH 6.8, followed by heating at 100 C for 17 minutes. Proteins were resolved electrophoretically, transferred to nitrocellulose and probed with monoclonal antibody 2-7A. This figure is representative of the binding pattern of other monoclonal and polyclonal antibodies that were tested against these viruses (see Table 5)

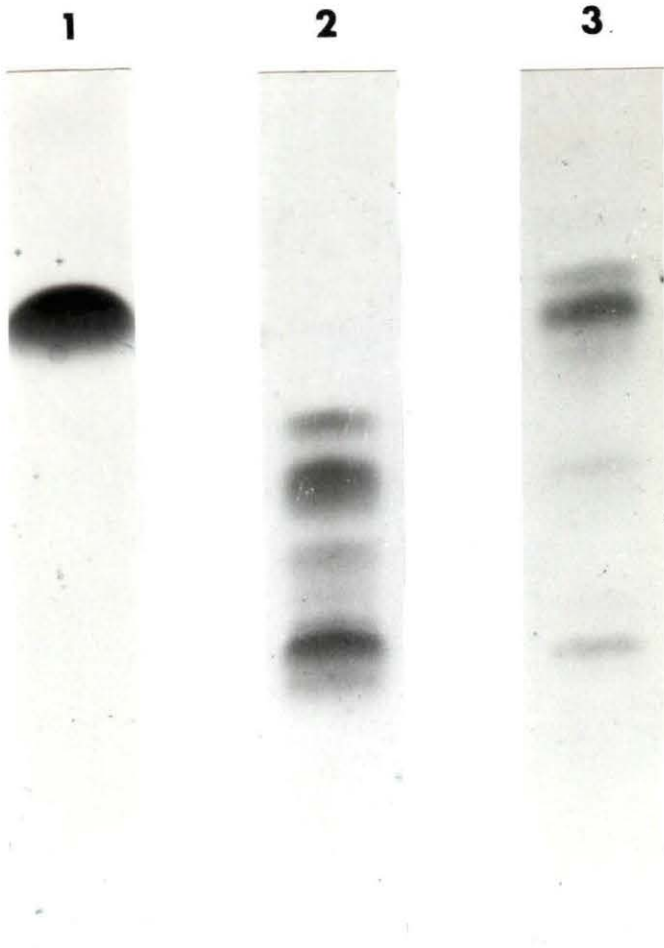


Figure 4. Immunoblot analysis of plant sap from uninfected cowpea (cv. California Blackeye) in lanes 1 and 2, diluted 1/5 and 1/25 in Tris-HCl containing 1% mercaptoethanol (2-MCE) and sodium dodecyl sulfate (SDS), respectively. Cowpea leaves were ground with a mortar and pestal in 0.125 M Tris-HCl containing 0.1% 2-MCE, pH 7.6. Antigens in the plant sap were denatured by adding an equal volume of 0.125 M Tris HCl containing 1% 2-MCE and SDS, pH 6.8, followed by heating at 100 C for 17 minutes. Proteins were resolved electrophoretically, transferred to nitrocellulose, and probed with IgM purified from cell line 4-5B





Figure 5. Immunoblot analysis of the molecular weight (MW) standards phosphorylase B, ovalbumin, soybean trypsin inhibitor and lysozyme. Molecular weight standards were diluted (1/20) with sample buffer and denatured by incubating at 100 C for 5 minutes. The standards were resolved electrophoretically, transferred to nitrocellulose and probed with monoclonal antibody 4-6F. This figure is representative of the binding pattern of several other monoclonal and polyclonal antibodies that were tested against and reacted with the molecular weight standards.

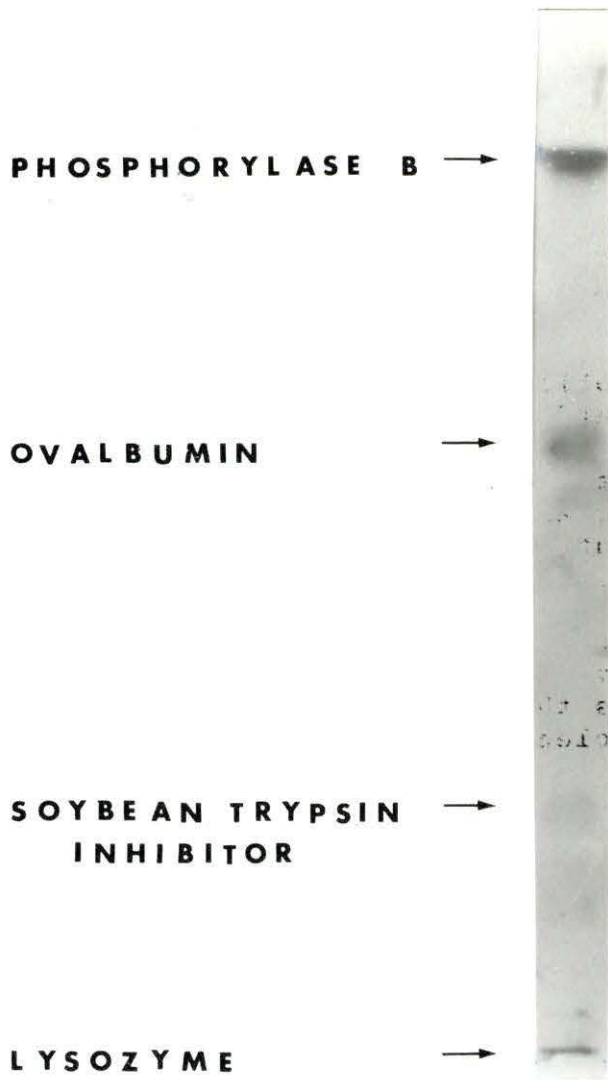


Figure 6. Reactions of purified IgM from cell line 4-5B with CPSMV II (B) in electron microscopy. Gold labelled goat anti-mouse IgM, which attached to antibody 4-5B (A), was used as the detection antibody

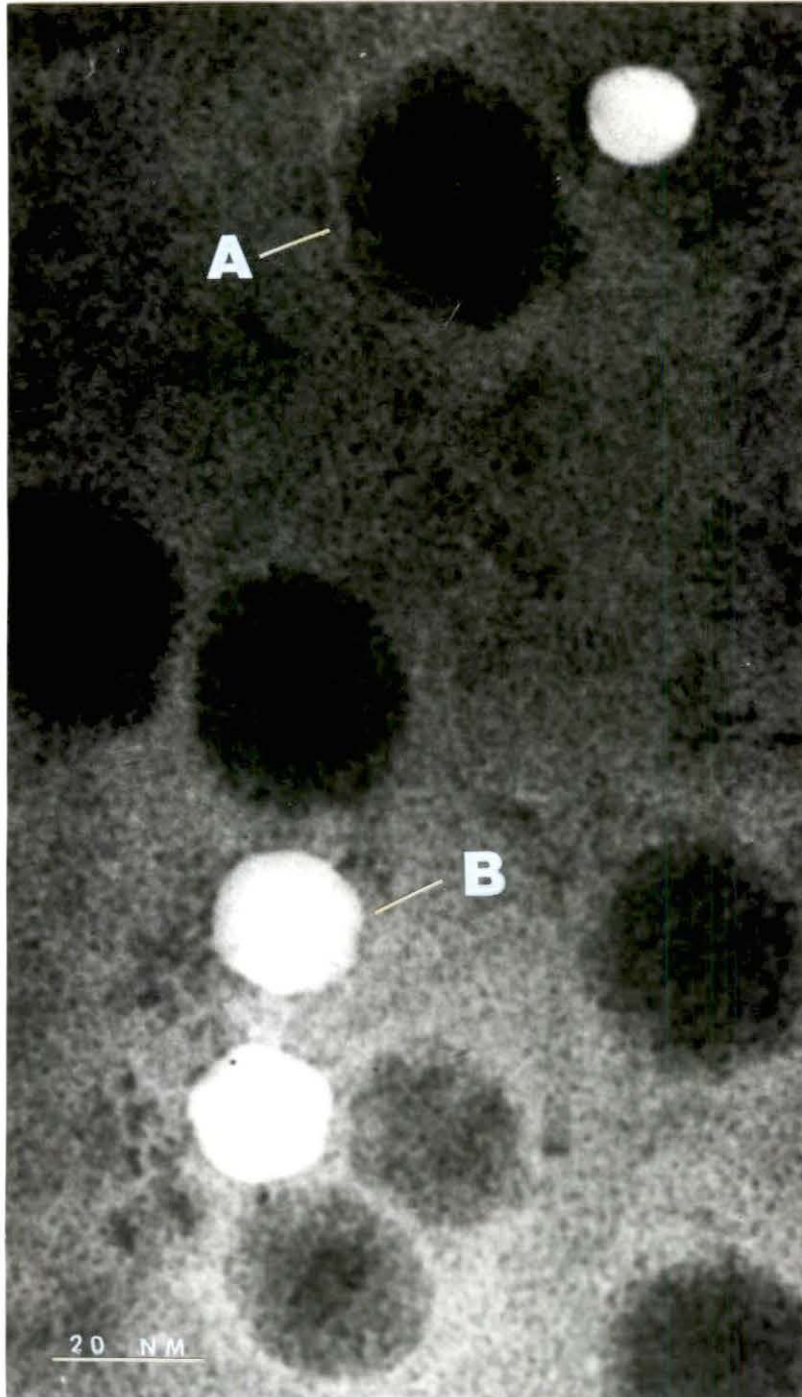


Figure 7. Reaction of purified IgM from cell line 4-5B in electron microscopy with SMV (B). Gold labelled goat anti-mouse IgM, which attached to antibody 4-5B (A), was used as the detection antibody

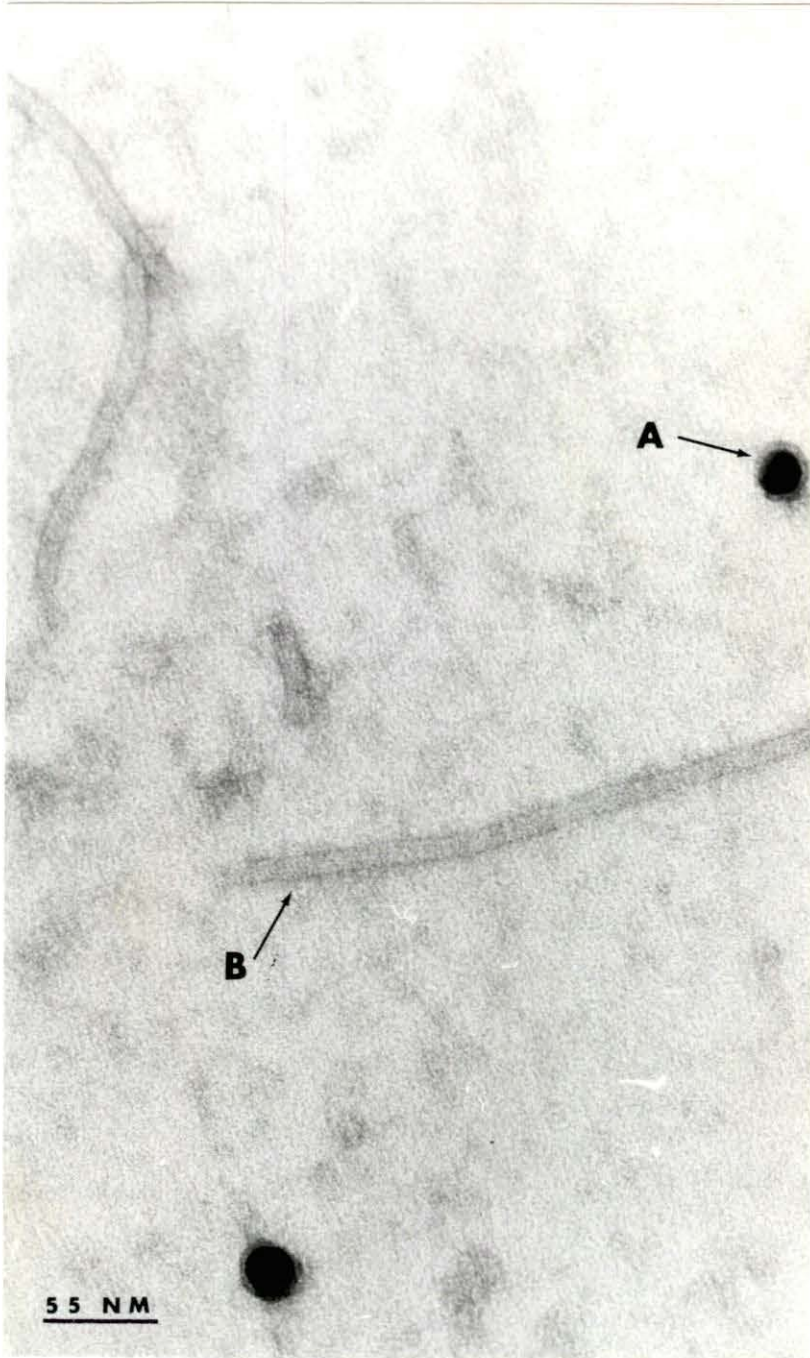


Table 6. Results of consecutive and simultaneous competition assays of monoclonal antibodies developed against CPSMV I and II as determined by indirect ELISA. CPSMV I concentrations of 4 ug/ml were used

Monoclonal antibodies	Competition <sup>a</sup>
1-9G	- <sup>b</sup>
1-11G	-
2-1F	+ <sup>c</sup>
2-7A	+
2-11A	+
2-11G	-
3-9C	+
3-11C	+
4-5B	+
4-5F	+
4-6F	-
B12	+
5B	+
G10	-
F1	+
6A	+
H6	+

<sup>a</sup>Competition was assumed not to have occurred if, in both the consecutive and simultaneous assays, the percent maximum absorbance values of the unlabelled monoclonal antibodies, at dilutions ranging from 1/1 (undiluted) to 1/1024 or 1/100,000, was greater than 75%.

<sup>b</sup>Monoclonal antibodies did not compete with H6.

<sup>c</sup>Monoclonal antibodies did compete with H6.

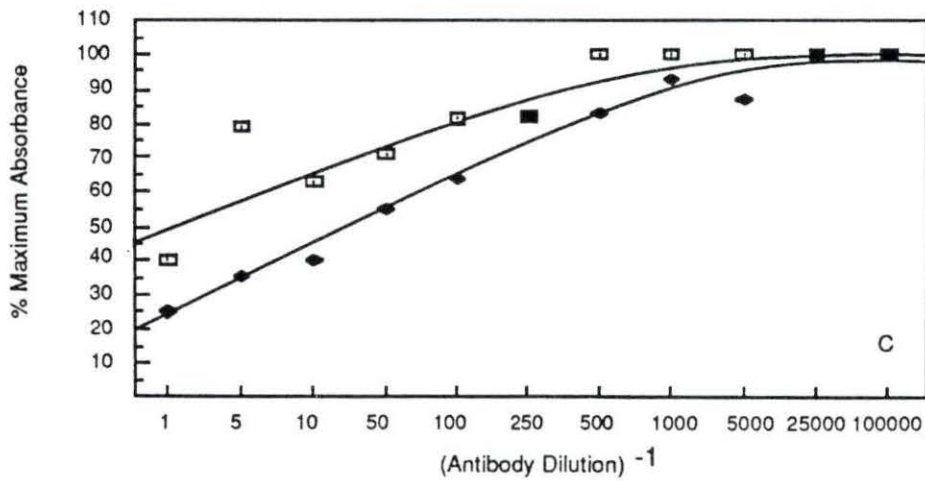
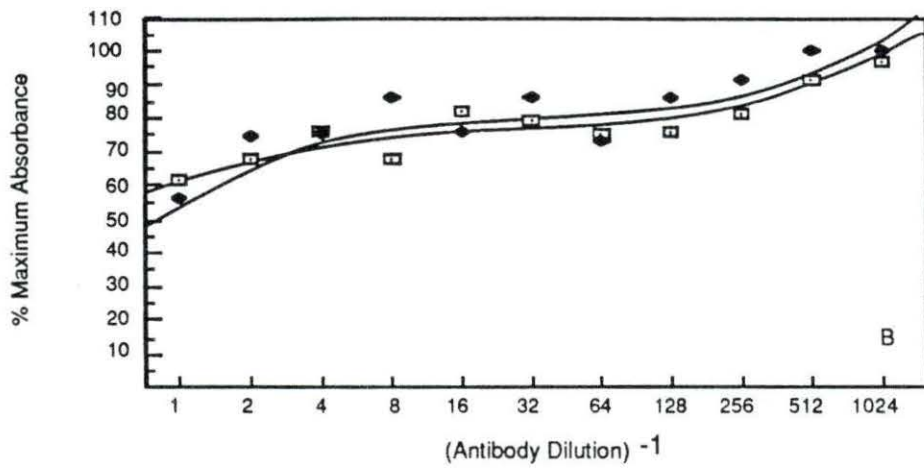
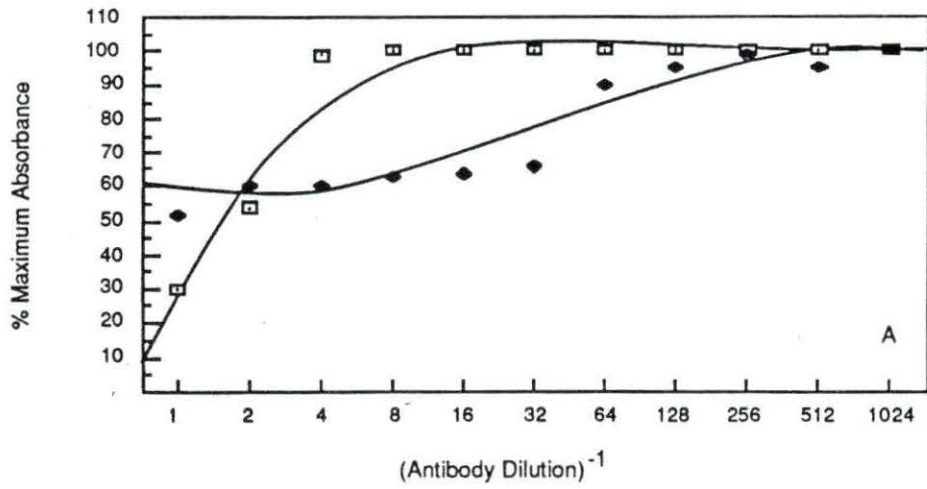


Figures 8 (A-Q). Dilution profiles of monoclonal antibodies developed against CPSMV I and II in competition assays with undiluted monoclonal antibody H6. CPSMV I (2 ug/ml) was used to coat the ELISA plate.

Graphs A-Q present results with the following monoclonal antibodies used in the competition assays:

A=4-5B, B=3-9C, C=5B, D=4-5F, E=2-1F, F=B12, G=6A, H=3-11C, I=2-7A, J=2-11A, K=F1, L=H6, M=2-11G, N=G10, O=1-9G, P=4-6F, and Q=1-11G.

Competition of the unlabelled antibodies was determined for the consecutive assay (unlabelled antibodies were added prior to the labelled antibody H6;  $\square$ — $\square$ ) and for the simultaneous assay (unlabelled and labelled antibody added together;  $\blacklozenge$ — $\blacklozenge$ ). The positive control (wells with labelled H6 with no unlabelled antibodies present) was designated as having an absorbance of 100%. The amount that each monoclonal antibody decreased the maximum (100%) absorbance of H6 was calculated at dilutions ranging from undiluted (1/1) to 1/1024 or 1/100,000 expressed as the reciprocal of the dilution. The assay procedure is described in materials and methods



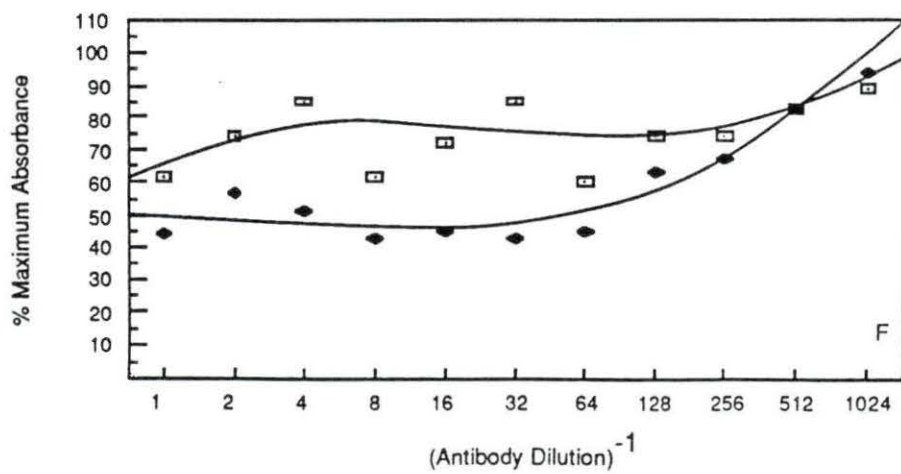
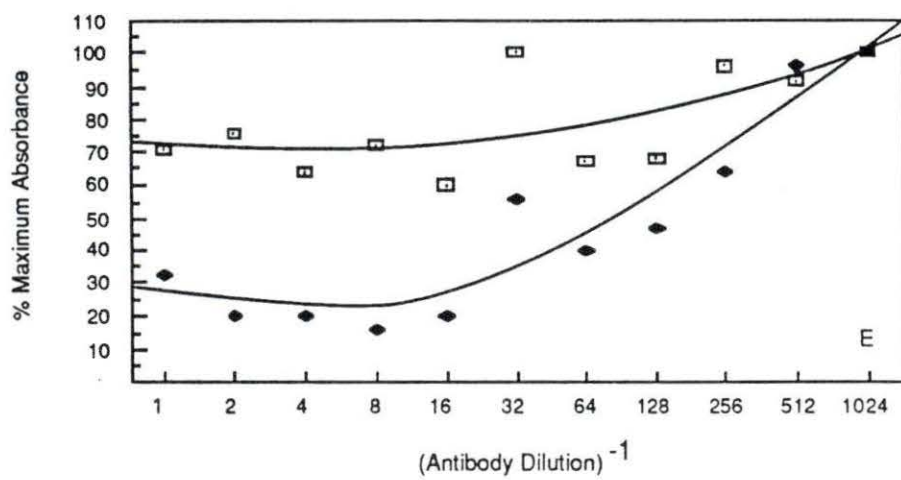
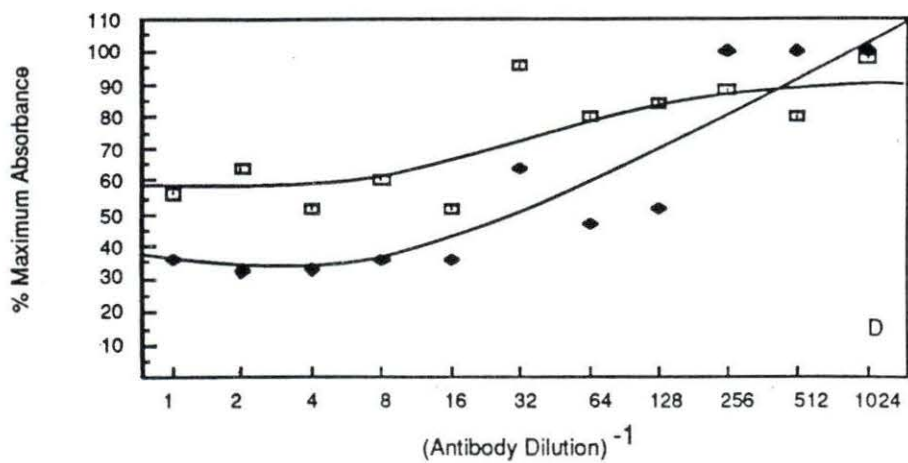


Figure 8 (continued)

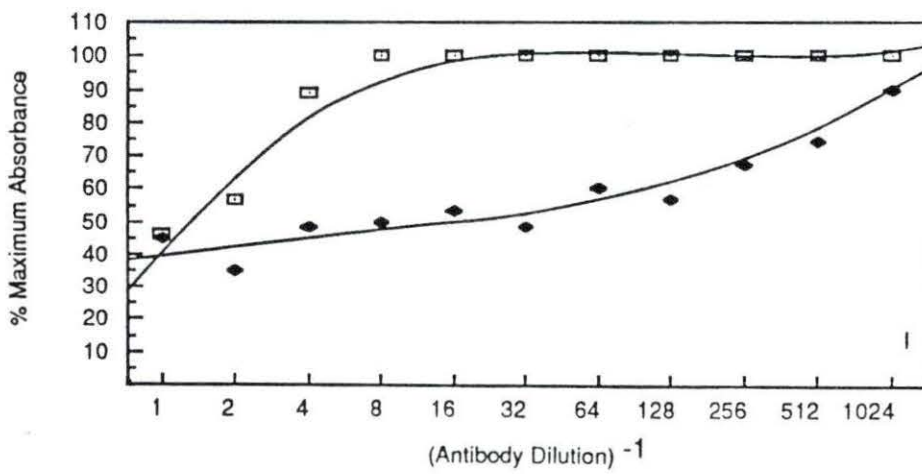
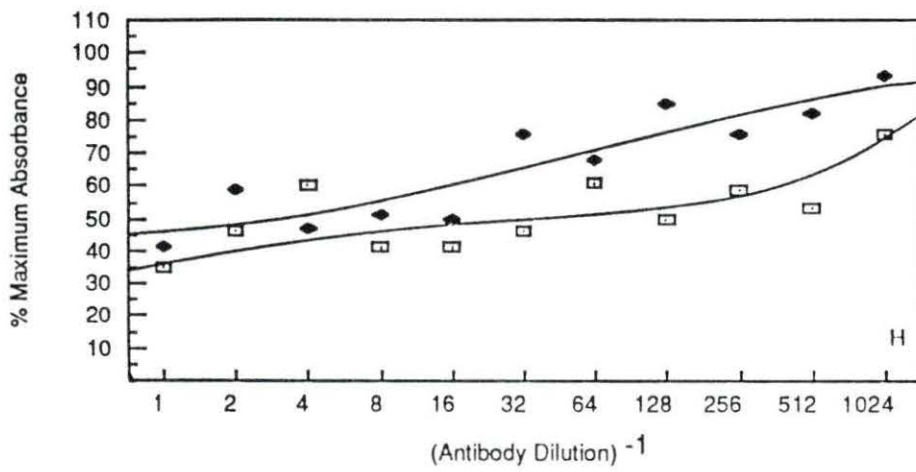
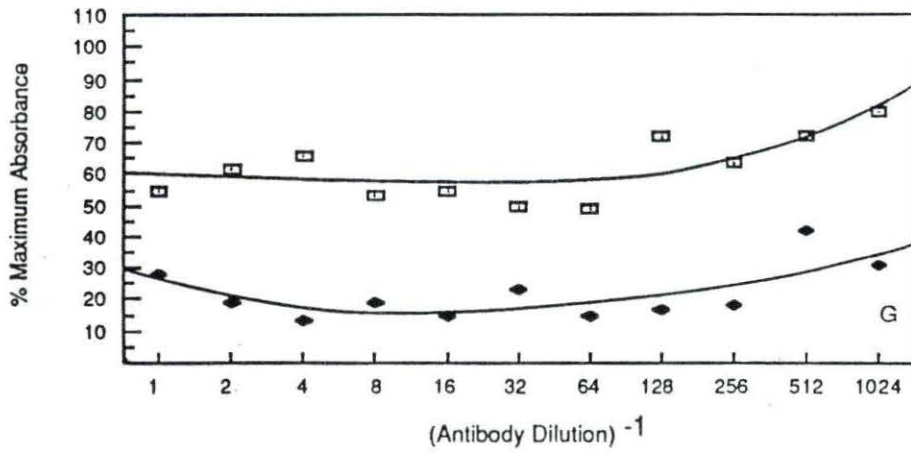


Figure 8 (continued)

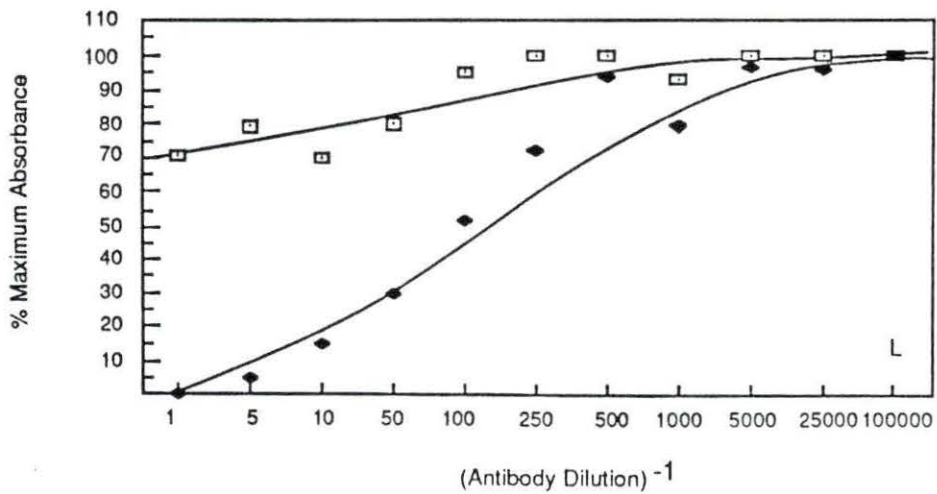
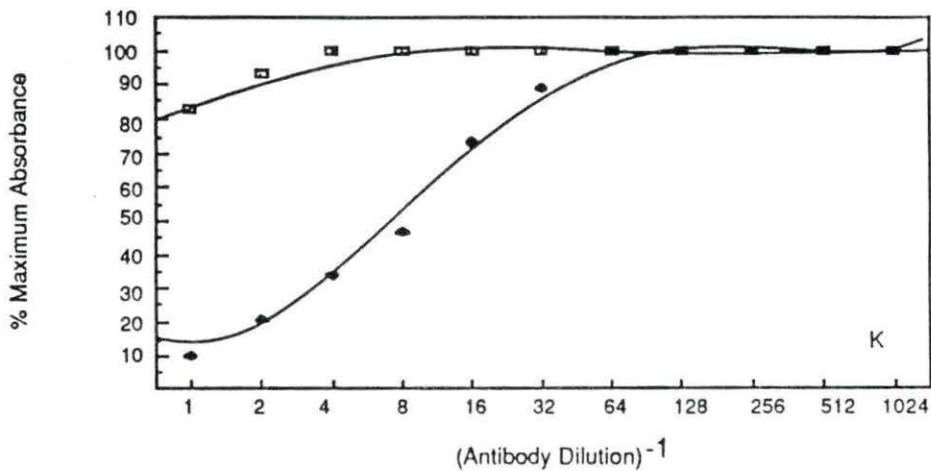
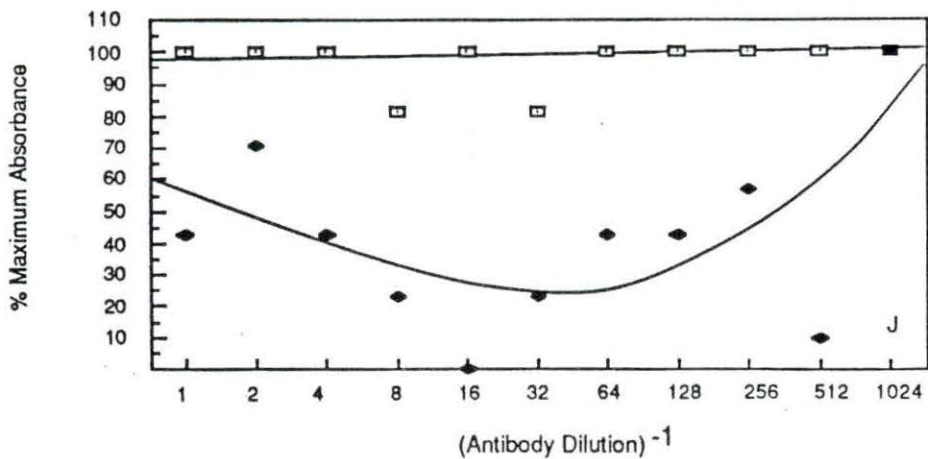


Figure 8 (continued)

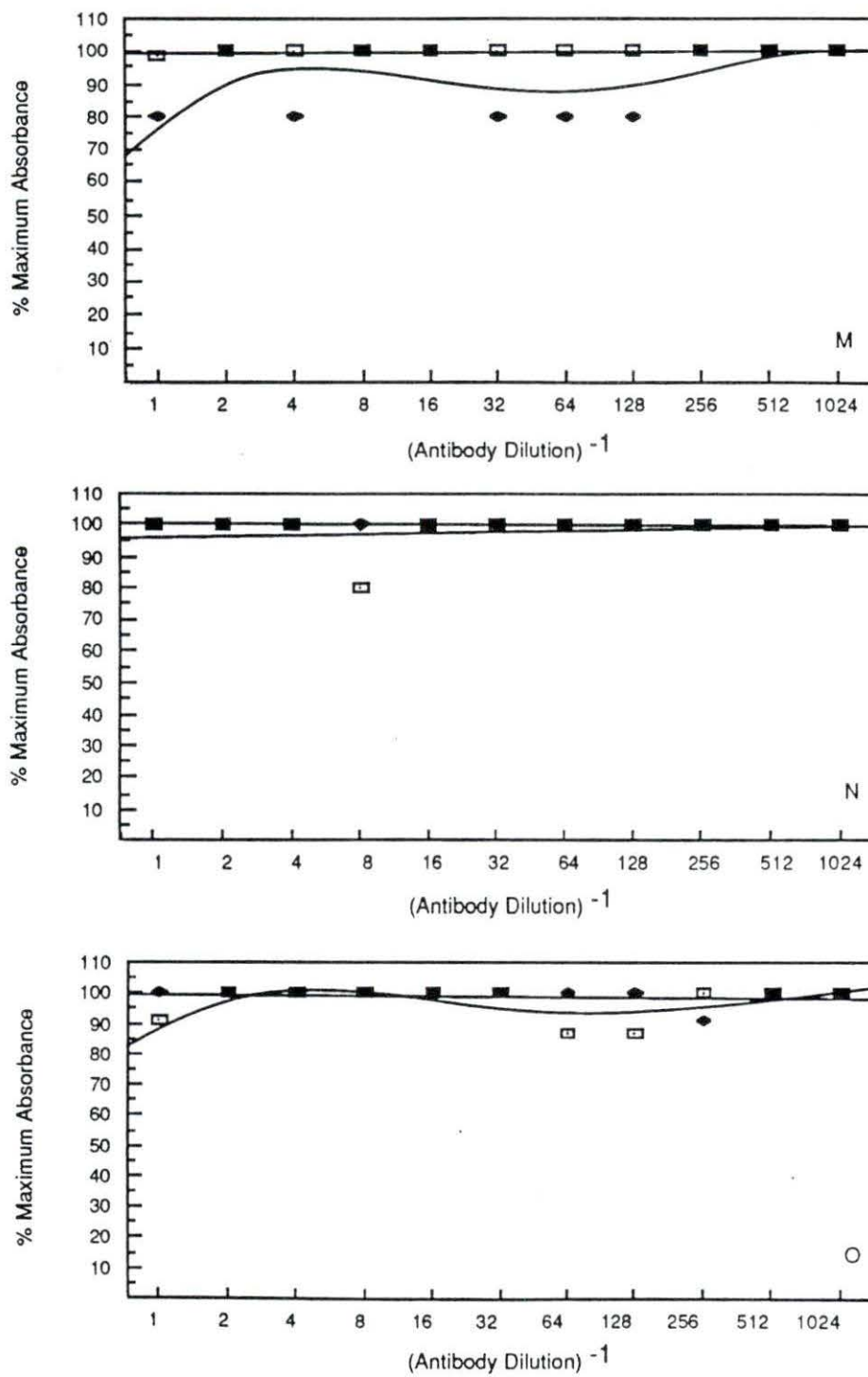


Figure 8 (continued)

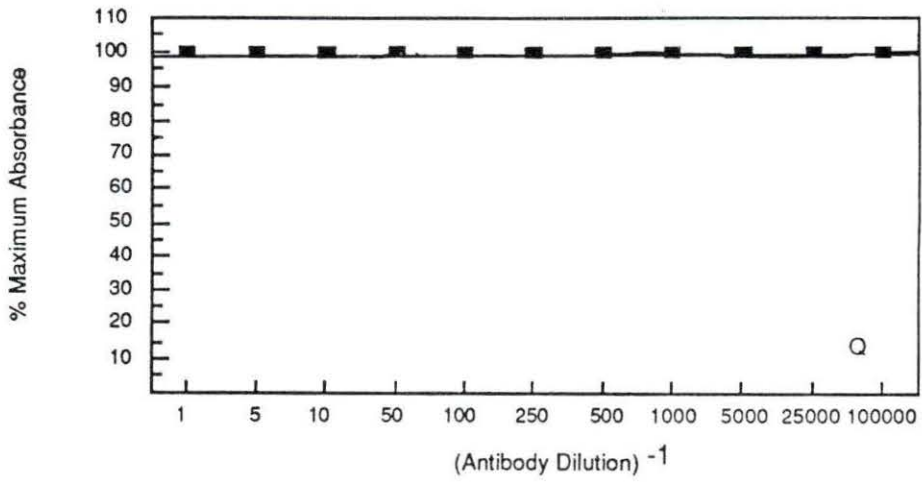
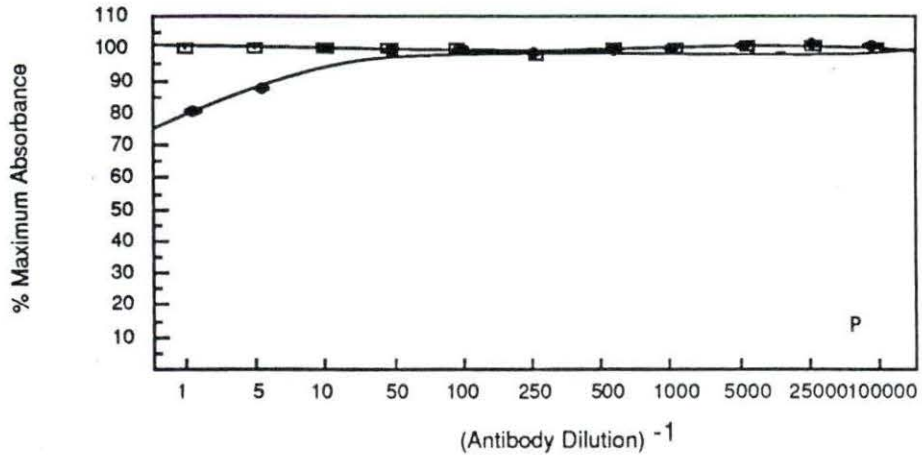


Figure 8 (continued)

## DISCUSSION

Monoclonal antibodies were produced to CPSMV II using a modification of methods described by Van Deusen and Whetstone (1981). Each fusion yielded numerous hybridoma cells. Cell lines that were cloned were selected by indirect ELISA. Many of the 45 hybrids that were cloned may have been outcompeted by non-producing variants or hybrids producing antibodies that were not specific for CPSMV II. Since chromosomal segregation is a frequent event with hybrid cells during the first few weeks following a fusion (Milstein, 1980; Williams et al., 1977), many hybrids that may have initially produced antibodies to CPSMV II ceased to do so. Cloned hybrids remained stable.

The most appropriate virus concentration, virus coating buffer and blocking formulation was determined for the indirect ELISA. Virus concentrations ranging from 0.5 to 20.0 ug/ml were compared. The most suitable concentration was 2 ug/ml since it resulted in the highest P/N ratio (Fig. 1).

The second parameter studied was the coating buffer formulation most appropriate for CPSMV II.  $\text{NaKPO}_4$ , pH 7.0; PBS, pH 7.5; and  $\text{NaCO}_3$ , pH 7.5 were compared. The results indicated that all could be used equally well in the indirect ELISA as a coating buffer.



Several blocking formulations were compared to see which most effectively prevented non-specific reactions. Blotto was chosen since it resulted in a sensitive assay and also yielded low background readings (Table 1). After the indirect ELISA was optimized, it was utilized to select hybridomas secreting antibodies specific for CPSMV II.

A modified indirect ELISA was used for isotyping 11 monoclonal antibodies produced against CPSMV II and 6 monoclonal antibodies produced against CPSMV I. Sixteen of the cell lines produced IgM antibodies and only one produced IgG2a antibody. The reasons for the high incidence of IgM producers is unclear although it have been a result of the immunization schedule of the mice or the use of Freund's complete adjuvant during immunization.

The purification of IgM has traditionally been difficult (Goding, 1983). IgM was, however, isolated using an anti-mouse IgM agarose column. IgM bound specifically to the column and was eluted by lowering the pH to 6.0. Two peaks were detected. The first one represented contaminants while the second one was the eluted IgM. Excellent yields were obtained; for example, 4.76 mg of antibody was purified from 250 ml of DMEM-R. Normally, antibodies are purified from ascites fluid because insufficient yields are obtained from growth medium. However,

the yield obtained indicated that IgM can efficiently be purified with this agarose column from DMEM-R medium as well. It can also be utilized to purify IgM from ascites fluid; the procedure used is identical to that described for DMEM-R, except significantly smaller volumes of ascites fluid are used.

Monoclonal antibodies should be characterized so that those that have desired characteristics can be identified and selected for various analyses such as diagnostic assays. Immunoblot analysis were originally conducted with the intent of characterizing monoclonal antibodies produced against CPSMV I and II by testing them against several comoviruses. However, the specificities of the antibodies could not be identified and therefore, they were tested against a greater panel of antigens. Once again, it remained difficult to characterize the antibodies; however, since the antibodies reacted with many of the antigens, the hypothesis evolved that the antigens may have common epitopes. Therefore, more immunoblot analyses were conducted, but this time with the intent of both characterizing the monoclonal antibodies produced against CPSMV I and II as well as other monoclonal and polyclonal antibodies, and determining whether there were any antigenic relationships among the plant and animal viruses and other antigens. Determining whether antigens

are related is of importance because relatedness would indicate that some antibodies can react with the antigens that have been identified as having common epitopes. Since antibodies are frequently employed in diagnostic assays for identifying the causal agent of a disease, it is important to know whether they are specific for the causal agent or whether they also react with closely related or supposedly unrelated antigens. Specific antibodies can be selected by testing a series of different antibodies against a panel of antigens that are known to be related and determining which of the antibodies reacts only with the causal agent. The assays indicated that the many of the monoclonal and polyclonal antibodies reacted with the antigens examined (Table 4). Several generalizations can be made. Most of the monoclonal antibodies reacted with CPSMV I, II, III, IV, BPMV, CPMV (Fig. 2), LMV (Fig. 3) and the molecular weight standards phosphorylase B, ovalbumin, soybean trypsin inhibitor and lysozyme (Fig. 5). Approximately one-half of the antibodies tested reacted with MDMV and BSMV (Fig. 3) although none did with SMV. Antibodies also reacted with NDV and with antigens from healthy cowpeas (Fig. 4).

Wedge and Svenneby (1986) and Spinola and Cannon (1985) believed that the blocking buffer could have an impact upon the binding patterns of antibodies. There-

fore, four different blocking formulations were compared: 3% BSA in Tris-saline, pH 10.2; 3% BSA in PBS, pH 7.5; Blotto; and 50% fetal bovine serum in PBS-Tween (Table 5). All yielded identical results; i.e., monoclonal antibody 4-5B reacted with CPSMV II and the molecular weight standards regardless of the blocking agent used. Thus, these formulations had little impact upon the binding of this antibody.

Johnson et al. (1984) used Blotto to dilute the primary antibodies for immunoassays and believed that it had an impact upon binding. Therefore, antibodies produced by cell line 2-7A were diluted in PBS-Tween with 1% BSA or PBS-Tween with 1% gelatin (Table 5). In both situations, the antibody did react with CPSMV II and thus this modification had little impact upon binding of monoclonal antibody 2-7A to this virus. In order to unequivocally conclude whether the buffers used to dilute the primary antibody did or did not have an impact upon the binding of this and the other antibodies utilized in the immunoblot analyses, it would be necessary to test all of these antibodies in the two buffers mentioned above, against the entire panel of antigens utilized in these assays.

The possibility also existed that the alkaline phosphatase conjugated goat anti-mouse IgM reacted non-

specifically with the antigens. Therefore, alkaline phosphatase from two commercial sources (Sigma and Cappel) were compared (Table 4). Neither reacted with CPSMV II. The alkaline phosphatase conjugate from Cappel was also tested against the molecular weight standards and did react with them. Thus, alkaline phosphatase conjugated goat anti-mouse IgM can react with some proteins other than mouse IgM.

Many of the antibodies reacted with numerous antigens in the immunoblot assays, and therefore, characterizing the antibodies and determining the antigenic relationships among the antigens remained difficult. Since this issue could not be resolved, the following hypotheses will be presented which may explain the reactions observed: (1) many non-specific protein-protein interactions took place, (2) the antigens used in the assays did share antigenic determinants, (3) the hybridoma cells were producing antibodies of more than one specificity, and/or (4) the antibodies bound non-specifically to the antigens due to the presence of calcium and magnesium in the buffer that the antibodies were diluted in. Studies that have been conducted in relation to the above hypotheses will now be discussed, and may indicate that one or a combination of these hypotheses could explain the reaction observed in the immunoblot assays.

Serological relationships and therefore shared antigenic determinants have been demonstrated to exist among four serotypes of CPSPMV, plant viruses from the same virus family, plant viruses from different viral families, and unrelated molecules. Lin et al. (1984) concluded that serotypes I, II, III, and IV of CPSPMV shared at least one antigenic determinant. Agrawal and Matt (1964) stated that CPMV, CPSPMV, BPMV and red clover mottle virus (all comoviruses) shared antigenic determinants. Barley stripe mosaic virus is distantly related to lychnis ringspot virus (both are hordeiviruses; Gibbs et al., 1963) Relationships among BPMV and the following comoviruses has been observed: CPMV (Shepherd, 1963), radish mosaic virus, squash mosaic virus (Cambell, 1964), red clover mottle virus (Gibbs et al., 1968) and pea green mottle virus (Valenta and Gressnerova, 1966). The serological relationships among comoviruses is represented in Figure 9 (Tomlinson, 1978). After studying this diagram, it is apparent that a rather complex pattern of relatedness exists and that many of these viruses do share epitopes. For example, CPSPMV is related serologically to andean potato mottle virus (Fribourg et al., 1977), BPMV (Cambell, 1964; Moore, 1973; Shepherd, 1963; Siler et al., unpublished observations cited in Tomlinson, 1978), bean rugose mosaic virus (Gamez, 1972), CPMV (Agrawal and Maat,

1964; Moore, 1973; Shepherd, 1963; Siler et al., unpublished observations cited in Tomlinson, 1978), pea green mottle virus (Gibbs et al., 1968), quail pea mosaic virus (Moore, 1973; Siler et al. unpublished observations cited in Tomlinson, 1978), radish mosaic virus (Cambell, 1964), red clover mottle virus (Valenta and Marcinka, 1971) and perhaps broad bean stain virus (Gibbs et al., 1968). Studies conducted by Shepherd (1963) indicate that there are serological relationships among CPMV, BPMV (both comoviruses) and tomato ringspot virus (a nepovirus). Querfurth and Berks (1976) found serological relationships among tobacco mosaic virus (TMV; a tobamovirus) and cocksfoot mild mosaic virus (CMMV; from the cocksfoot mild mosaic virus group), Molonia streak virus (MSV) and CMMV, and, TMV and MSV. A monoclonal antibody 41-21 that was produced against T lymphoma cells reacted with the  $V_k$  (variable region of kappa light chain) antigenic determinant of TEPC15 mouse myeloma protein and the Thy 1 antigen which is found on all T cells (Pillemer and Weissman, 1981). Monoclonal antibodies also cross-react with tropomyosin and vimentin, both cytoskeletal proteins (Blase et al., 1983).

Sperling et al. (1983) demonstrated, using radioimmunoassays, that antibodies are capable of binding to the immunogen as well as unrelated antigens. For example,

when mice were immunized with DNP-BGG (2,4-dinitrophenyl derivatives of bovine gamma globulin), the antisera collected reacted with DNP-BSA (BSA=bovine serum albumin fraction V), Ars-RSA (p-azobenzenearsonate derivative of rabbit serum albumin), Fl-Ova (fluorescein conjugated ovalbumin) and Dan-HSA (1-dimethylaminoaphthalene-5-sulfonyl derivative of horse serum albumin). They stated that the DNP-BGG antiserum bound to the unrelated antigens differently than with the DNP-BGG antigen. Moreover, they did not regard the binding of the antibodies to the antigens as completely "non-specific".

Fox and Siraganian (1986) tested the reactivity of 31 purified monoclonal antibodies with 10 unrelated proteins. Seventy one percent of the antibodies reacted with at least one of the dissimilar antigens. Only 29% of the monoclonal antibodies did not react with any of the nonhomologous antigens. Twenty-six percent of the antibodies reacted with lysozyme, 30% reacted with ovalbumin, and 10% reacted with soybean trypsin inhibitor. In the immunoblot assays, the antibodies reacted with lysozyme, soybean trypsin inhibitor, and ovalbumin as well (Table 5 and Fig. 6). They also reacted with phosphorylase B; however, this enzyme was not included in the panel of antigens compared by Fox and Siraganian (1986). The studies by Fox and Siraganian (1986) offer new



evidence that, in the immunoblot analyses, the antibodies may have reacted with unrelated antigens. These examples demonstrate that monoclonal antibodies have already been found that cross-react with unrelated antigens, probably because the antigens have common epitopes (Kearney, 1984). These studies suggest that the unrelated viruses and antigens used in the immunoblot assays may possibly have common antigenic sites. However, it is also possible that cells were producing antibodies of more than one specificity or that the antibodies were reacting non-specifically because of the presence of calcium or magnesium in the buffers used to dilute the antibodies in.

Liacopoulos et al. (1971) also reported that antibodies bound to dissimilar antigens; however, for reasons other than those cited above. They injected mice with sheep and pigeon erythrocytes and monitored the specificities of antibody producing cells using the method of immuno-cyto-adherence. Cells capable of secreting two distinct antibody molecules were seen. Hemolytic plaque forming cells (PFC) that reacted to one or two determinants appeared when mice were immunized with two unrelated antigens. This occurred because these PFCs produced two antibodies (Liacopoulos et al., 1976). These conclusions were also reached by Couderc et al. (1977). Thus, it seems plausible that the production of antibodies of

different specificities from one cell line could result in the cross-reactivities sometimes observed among unrelated antigens.

The specificity of some antibodies can be affected by calcium and magnesium. These ions can make some antibodies react non-specifically with antigens; however, if ethylenediaminetetraacetic acid (EDTA) is added, it can chelate these divalent cations (Linstromberg and Baumgarten, 1978). These ions can then no longer interfere with the binding of antibodies to an antigen and non-specific reactions may be eliminated. Kilpatrick et al. (1982) produced a monoclonal antibody against human CRP (C-reactive protein), a structure on human peripheral blood monocytes, which bound to CRP and a large variety of human and mouse cell types with filamentous intracellular structures. However, the binding of this antibody was thought to be controlled by the presence of  $\text{Ca}^{2+}$  since it only recognized calcium-associated epitopes (Kilpatrick et al., 1982). The antibodies utilized in the immunoblot assays were diluted in a buffer (TBS-Tween) that contained 0.001% calcium and magnesium but no EDTA. This may have caused the antibodies to bind non-specifically to the antigens and may explain the numerous reactions among the monoclonal and polyclonal antibodies, and the antigens.

Diacco et al. (1986) indicated that barley yellow

dwarf isolates could be screened using serologically specific electron microscopy. Immunosorbent electron microscopy was utilized to detect plum pox virus and apple chlorotic leaf spot virus (Kerlan et al., 1981). Therefore, electron microscopy is a useful tool for detecting plant viruses and for determining whether viruses are related. Electron microscopy was utilized to determine whether CPSMV II and SMV were related serologically. Results indicated that purified IgM from cell line 4-5B bound to CPSMV II but not to SMV (Figs. 7 and 8). This confirmed conclusions obtained by immunoblot analysis where identical reactions were observed (Table 5). Both electron microscopy and immunoblot assays indicated that CPSMV II and SMV do not share the epitope for antibodies produced by cell line 4-5B. However, to prove unequivocally that monoclonal antibody 4-5B was reacting serologically with CPSMV II but not with SMV the following assays would have to be undertaken. Monoclonal antibody 4-5B would have to be incubated with SMV and CPSMV II separately (as was done), and also with both viruses mixed together. If the antibody bound with CPSMV II but not with SMV when they were mixed together in addition to when they were separate, this would suggest that monoclonal antibody 4-5B reacted serologically with CPSMV II but not with SMV. Moreover, it would be interesting to perform

electron microscopy studies with all of the other antibodies utilized in the immunoblot assays and to observe whether these antibodies bind specifically to unrelated antigens.

In summary, the immunoblot analyses could not be utilized to characterize the monoclonal antibodies produced to CPSMV I and II or to determine the antigenic relationships among the plant viruses and other antigens. These, as well as other studies conducted by other investigators, suggest that any of the following hypotheses still seem possible: (1) many non-specific protein-protein interactions took place, (2) the antigens used in the assays did share antigenic determinants, (3) the hybrid cells produced antibodies of more than one specificity, and (4) the antibodies bound non-specifically to the antigens because of the presence of calcium and magnesium in the buffer that the antibodies were diluted in. Whether one or a combination of several of the hypotheses explains the cross-reactions observed in the immunoblot assays remains unresolved. Since the immunoblot assays indicated that the monoclonal antibodies produced to CPSMV II may not have been specific antibodies, suggestions will be made of what could be done, in the future, to aid in producing of monoclonal antibodies that are more selective, i.e., that react with only one or a few related

proteins.

Monoclonal antibodies are frequently made to a dominant epitope on an antigen. The antibodies produced to CPSMV II (as well as the other viruses utilized in the immunoblot assays) reacted consistently with the molecular weight standards lysozyme, ovalbumin, soybean trypsin inhibitor, and phosphorylase B. The monoclonal antibodies may have been produced to a dominant epitope on CPSMV II that was also shared by these antigens. By inducing a state of tolerance in mice to the dominant epitope, it may be possible to produce a more diverse panel of monoclonal antibodies because they may be produced to minor antigenic determinants rather than just the dominant determinant. This may be accomplished by injecting the molecular weight standards into a mouse a few hours after it was born followed by an injection with CPSMV II 15-20 weeks later. Columbeski and Dimond (1986) stated that 70% of the cell lines produced from mice that had been immunized using the conventional multidose immunization schedule (two peritoneal injection followed by two intravenous injections or modifications of this) produced antibodies that recognized a shared, immunodominant epitope. However, mice that had been tolerized with the dominant epitope resulted in the isolation of nine cell lines, none of which reacted with the dominant epitope.

Thorpe et al. (1984) compared mice that had been immunized using the single shot intrasplenic injection technique versus mice that had been immunized using the conventional multidose injection schedule (similar to the one described above). They concluded that the intrasplenic technique resulted in six cell lines that produced completely specific monoclonal antibodies against fibrin degradation products. However, using the conventional schedule, all 42 of the monoclonal antibodies obtained cross-reacted with non-crosslinked fibrin degradation products and fibrinogen. Perhaps, if the intrasplenic immunization technique was utilized with CPSMV II rather than the multidose schedule (as was done), specific monoclonal antibodies that only react with CPSMV II could be produced.

Mosmann et al. (1980) stated that the specificities of five monoclonal antibodies produced to chicken red blood cells (CRBC) were modified by changing the conditions of an assay such as temperature or pH. They demonstrated that monoclonal antibody CH-4 (an antibody produced against CRBC's) became totally specific for the  $B^2/B^2$  CRBC'S (one genotype of chicken erythrocytes) at 37 C, whereas the specificity of monoclonal antibody CH-20 did not change at temperatures ranging from 4 to 37 C. Furthermore, antibody CH-4 reacted with  $B^2/B^2$  CRBC's at pH

values ranging from 6.0 to 8.5 but not with B<sup>13</sup>/B<sup>13</sup>, B<sup>14</sup>/B<sup>14</sup>, or B<sup>21</sup>/B<sup>21</sup> (all are genotypes of chicken erythrocytes), whereas monoclonal antibody CH-20 was almost completely specific for B<sup>13</sup>/B<sup>13</sup> CRBC'S at a pH of 6.5. At a pH of 7.5, it also reacted with B<sup>15</sup>/B<sup>15</sup> and B<sup>21</sup>/B<sup>21</sup>. The specificities of the monoclonal antibodies produced to CPSMV II may be improved as well if the temperature and pH of the immunoblot assays were modified.

The purification process utilized to isolate CPSMV II may have resulted in virus contaminated with plant proteins from cowpeas. The mice would then have been immunized with the virus and the plant proteins. Cell lines may have been selected that secreted antibodies that bound to epitopes common to both of these antigens as well as other proteins. In order to be certain of the purity of CPSMV II, the following protocol could be utilized: electrophorese the virus on polyacrylamide gel, cut out the viral bands and elute out the virus for use in the immunization process. This may result in the production of monoclonal antibodies specific to CPSMV II.

Vaidya et al. (1985) screened hybridomas produced against creatine kinase and lactate dehydrogenase by indirect ELISA and by solution phase radioimmunoassay (RIA). They stated that the indirect ELISA may be inappropriate for determining useful monoclonal antibodies

because the conformation of creatin kinase and lactate dehydrogenas may be changed when they bind to a solid phase. Therefore, they felt that the solution phase radioimmunoassay may be a more effective screening assay. The monoclonal antibodies produced against CPSMV II were selected by indirect ELISA. The conformation of CPSMV II may have been modified when it bound to the microtiter plates which may have resulted in the production of antibodies that could react with many dissimilar antigens that shared common epitopes. The double antibody sandwich ELISA may be a better assay to use in the selection process since the antigen is not bound directly to the ELISA plate in this assay. Instead, a capture antibody is bound to the plate and the virus, in turn, is bound to this antibody. In this situation, the conformation of the antigen may remain intact and monoclonal antibodies that are more specific may be produced.

The monoclonal antibodies used in immunoblot assays were screened using the indirect ELISA. However, it has been suggested that the design of the assay for which antibodies will be utilized, should also be employed in the screening process. Therefore, monoclonal antibodies to be used in immunoblot assays should be screened with this assay which may result in the production of more selective monoclonal antibodies.



The possibility exists that if one or a combination of these suggestions were considered, in the future when producing monoclonal antibodies to CPSMV II, antibodies specific for CPSMV II could be produced. The strategies described may also be useful for others involved in the production of monoclonal antibodies to plant viruses or other antigens.

The results of the immunoblot assays remained equivocal since the many of the antibodies reacted with numerous viruses and other antigens. As a result, an alternative approach was employed for characterizing antibodies against one of the antigens (CPSMV I).

Monoclonal antibodies produced to CPSMV I and II were characterized in simultaneous and consecutive competition assays. This was accomplished by determining which of the monoclonal antibodies competed with the biotinylated antibody H6, whether the affinity or the concentration of each of the antibody had a greater impact upon their ability to compete, and whether the antibodies competed for one or for two binding sites on the CPSMV I antigen. Selected antibodies that differed the most extensively in the above characteristics could be utilized, in the future, to study the topographical arrangement of epitopes (which is the demonstration of antigenic differences among proteins; Yewdell and Gerhard, 1981) of different

serotypes of CPSMV.

The biotin/avidin system can be used in many different types of assays. It was originally described by Bayer and Wilchek (1979) for immunochemical staining. Guesdon et al. (1979) utilized biotin and avidin in enzyme-immunoassays to increase their sensitivity. They also studied the effects of introducing biotin into antibody molecules and concluded that ability to bind antigens was not reduced even when amino groups on the antibody molecule were extensively substituted with biotin. The biotin/avidin system offers several other advantages: (1) biotin can be attached to small and large molecules under mild conditions, (2) biotin and avidin are both relatively inexpensive, (3) they are stable, and (4) they have a high binding affinity (Bayer et al., 1979).

The biotin/avidin system was utilized in competition assays for many of these reasons. However, before these assays could be performed, the most appropriate concentration of the virus, biotinylated antibody, and avidin were determined. Results indicated that the most suitable concentrations of these parameters were 4, 2.5, and 3 ug/ml.

Once the assay had been optimized, the competition assays were performed. The dilution profiles of the 17 monoclonal antibodies (Fig. 8 A-Q) suggests that the

monoclonal antibodies can be divided into two basic categories: (a) those that did compete, and (b) those that did not compete. Antibodies that did not compete included: 2-11G, G10, 1-9G, 4-6F, and 1-11G (Fig. 8 M-Q). The remaining antibodies did compete (Fig. 8 A-L). A summary of competing and non-competing antibodies is shown in Table 6.

The dilution profiles of the simultaneous and consecutive assays also indicate whether the ability of an antibody to compete is influenced more by its affinity or by its concentration. Simultaneous assays are primarily affected by the concentration of the unlabelled antibody (Berzofsky and Berkower, 1984). In this assay the unlabelled and labelled antibodies are added together and compete simultaneously for binding sites on the antigen (Cherel et al., 1985; Kalmakoff et al., 1977). At high concentrations (low dilutions), more of the unlabelled antibody will bind and concurrently, more competition will occur (Kalmakoff et al., 1977). As the concentration of the unlabelled antibody decreases (dilution increases) fewer of these antibodies bind. As a result, more of the biotinylated antibodies bind, and the unlabelled antibody competes less with the labelled antibody. The affinity of the unlabelled is also important in the simultaneous assay (Berzofsky and Berkower, 1984). If the affinity of the

antibody is low, less of the unlabelled will bind. Assuming the affinity of the biotinylated antibody is higher, more of it will bind since the unlabelled antibody competes only slightly with it. A high affinity antibody will compete by approximately 50% if the biotinylated antibody is also of high affinity. This occurs because both antibodies have equal chance of binding to the epitopes on the antigen.

The consecutive assay relies primarily upon the affinity of the unlabelled antibody (Berzofsky and Berkower, 1984; Hosang, 1985). In the consecutive assay, the unlabelled antibody is added to the wells prior to the addition of the biotinylated antibody. Since the unlabelled antibody does not have to compete with the biotinylated antibody, it can bind to all of the available sites on the antigen. When the biotinylated antibody is added it may replace unlabelled antibodies that dissociate from the binding sites. The dissociation of an unlabelled antibody depends upon its affinity, which in turn affects its equilibrium equation. The equilibrium equation for an antigen-antibody reaction is the same as for any reversible bimolecular binding reaction. The equation for chemical equilibrium is:  $S + L \rightleftharpoons SL$  where S=antibody binding sites, L=antigen binding sites, and SL=the complex of the two (Berzofsky and Berkower, 1984). If the af-

finitly of the unlabelled antibody for the antigen is low, the equilibrium equation is in favor of the dissociation of the antibody. The biotinylated antibody will replace the unlabelled antibody, if the labelled antibody's affinity for the antigen is higher. In this situation, there is little competition by the unlabelled antibody. On the other hand, if the affinity of the unlabelled is high, the equilibrium equation is in favor of the unlabelled remaining bound, and therefore, only a small percentage of the binding sites are replaced by the biotinylated antibodies. In this situation, the unlabelled antibody competes to a significant degree with the labelled antibody.

To ascertain whether the concentration or the affinity of each monoclonal antibody affected its ability to compete with the biotinylated antibody, the consecutive and simultaneous assay curves were analyzed in the following manner. The slopes and the percent maximum absorbance of both assays were considered to determine whether the concentration or the affinity of an antibody had a larger impact upon its ability to compete. If the slope of the consecutive assay curve was steep, the data suggest that the antibody had a low affinity for the epitopes and thus the affinity of the antibody had little influence upon its ability to compete. On the other hand, if the slope of

this curve was relatively shallow or increased gradually, the antibody had a high affinity for the antigen and therefore the affinity of the antibody did affect competition. The simultaneous assays was examined in the similar manner. A steep slope indicated that the concentration of the antibody did not have a large impact upon the ability of the antibody to compete whereas a shallow slope suggested that the concentration did have an influence upon competition by the antibody. The percent maximum absorbance values of both the consecutive and simultaneous assays, at all dilutions (but especially dilutions ranging from 1/1 (undiluted) to 1/500, since there appeared to be a sufficient quantity of antibody available at these dilutions to affect competition), were also analyzed. If the percent maximum absorbance was lower for the consecutive assay, affinity was considered to have a greater impact upon the ability of an antibody to compete with the biotinylated antibody, whereas if the percent maximum absorbance of the simultaneous assay was lower, the concentration was thought to affect competition more. In summary, the percent maximum absorbance and slopes of both the simultaneous and consecutive assays were considered for each antibody, when deciding whether the concentration, affinity or both affected competition.

Monoclonal antibody H6 (Fig. 8L) is homologous to the

biotinylated antibody H6. The concentration of this antibody affects its ability to compete more than its affinity does. This is apparent because the unlabelled antibody completely inhibits the binding of the labelled antibody at high concentrations (low dilutions, i.e., 1/1 dilution). Furthermore, the percent maximum absorbance of the simultaneous assay curve is from 5 to 70% lower than that of the consecutive assay, at dilutions ranging from 1/1 to 1/1000. The consecutive assay indicates that this is a low affinity antibody since the antibody does not compete well even at low dilutions (high concentrations), i.e., the percent maximum absorbance ranges from 71 to 100% at dilutions ranging from 1/1 to 1/1,1024, respectively. This antibody may have such a low affinity for CPSPMV I for the following reasons. The unlabelled antibody utilized in the competition assays was not purified from culture media. Non-specific protein-protein interactions, which decreased the apparent affinity of the antibody, may have occurred among other proteins in the media and the unlabelled antibody H6. Therefore, as indicated in the consecutive assay curve, the affinity of this antibody had little impact upon its ability to compete with the biotinylated antibody.

The monoclonal antibodies 4-5B and 2-7A (Fig. 8 A and I) are low affinity antibodies as is apparent from the

shape of their consecutive assay curves. The slope of these curves is very steep between dilutions of 1/1 and 1/4 which indicates that the equilibrium equation of these antibodies is in favor of dissociation. As a result the unlabelled antibodies were rapidly replaced with the biotinylated antibody. The concentration of the unlabelled antibody had a larger impact upon its ability to compete with the labelled antibody. This is indicated in the curve of the simultaneous assays, where there is a gradual decrease in competition as the concentration of the unlabelled antibodies decreases. This suggests that, while there is a sufficient concentration of unlabelled antibodies present, these antibodies can inhibit the binding of the labelled antibody. Furthermore, the percent maximum absorbance of the simultaneous assay curves is lower than the consecutive assay curves which also indicates that the concentration of the unlabelled antibodies had a greater impact than the affinity upon competition. These are low affinity antibodies for the same reason as described for antibody H6.

The concentration of monoclonal antibodies 5B, 4-5F, 2-1F, B12, 6A, 2-11A, and F1 (Fig. 8 C-G, J and K) influenced their ability to compete more than their affinity did. That the affinity affects competition more than antibodies 4-5B and 2-7A is evident because of the in-



clination of the consecutive assay curves. These curves have relatively gradual upward slopes (as compared to Fig. 8A), which indicates that the equilibrium equation is in favor of the unlabelled antibodies. The concentration of these antibodies is important as well which is demonstrated by the shape of the simultaneous assay curves. Therefore, as the concentration of the unlabelled antibodies decreases (dilution increases), more of the biotinylated antibody binds and competition by the unlabelled antibodies decreases. However, since the percent maximum absorbance of the simultaneous curves is lower than that of the consecutive assay curves, the concentration of these antibodies had a greater impact upon their ability to compete than their affinity did and the interpretation of the data may be made in a manner similar to that of antibody H6.

Alternatively, the consecutive assay for monoclonal antibody 3-11C (Fig. 8H) suggests that the affinity of this antibody had a greater impact upon its ability to compete than did its concentration, and that the equilibrium equation was in favor of the binding of the labelled antibody. The gradual increase in the slope of the simultaneous assay indicates that the concentration was also important; as the concentration of the unlabelled antibody decreased (dilution increased) competition

decreased. However, because the consecutive assay was relatively flat, and since the percent maximum absorbance of this curve is from 10-40% lower at all dilutions, the affinity had a greater impact than the concentration upon competition. The affinity of this antibody may have a significant impact upon its ability to compete for the following reason. Proteins in the flask media may have bound non-specifically to the antibodies; however, because the antibodies had a low affinity for the proteins in the media but had a high affinity for the antigen (CPSMV I), the equilibrium equation may have been in favor of the antibodies dissociating from the proteins in the flask media, and then binding specifically to CPSMV I.

Monoclonal antibody 3-9C (Fig. 8B) appears to be a high affinity antibody because the slope of the consecutive assay increases very gradually. This curve suggests that as the dilution of the antibody increases, the equilibrium equation remains in favor of the unlabelled antibody because of its high affinity. Therefore, this antibody remains bound and continues to compete with the biotinylated antibody. The slope of the simultaneous assay curve is also relatively level which indicates that the concentration of the unlabelled antibody is important as well. The unlabelled antibody can continue to compete with the labelled antibody at high dilutions such as

1/500. Above this dilution, there is an insufficient concentration of the unlabelled antibody present to prevent the labelled antibody from binding to virtually all of the binding sites. The consecutive and simultaneous assay curves also indicate that since the percent maximum absorbance of both assays is very similar at all dilutions, both the concentration and affinity of this antibody have approximately equal impact upon its ability to compete. The affinity of this antibody had an impact upon competition for the same reason as described for antibody 3-11C.

The assumption was made that no competition occurred if the percent maximum absorbance in both the simultaneous and consecutive assays was above 75%, at all dilutions. Since all of these parameters were fulfilled, the remaining antibodies (2-11G, G10, 1-9G, 4-6F, and 1-11G, Fig. 8 M-Q, respectively and Table 6) did not compete with the biotinylated antibody.

The dilution profiles of the monoclonal antibodies also suggested whether the unlabelled antibodies competed for one or for two sites (Berzofsky and Berkower, 1984). When the consecutive and/or simultaneous assay curves were diphasic the antibodies probably bound to two sites; whereas, if both the consecutive and simultaneous assay curves were monophasic the antibodies competed for one

site. The antibodies that competed for two sites may have done so for several different reasons. (1) The antibodies had a higher affinity for one of the two epitopes. They would bind first to the site for which they had a higher affinity, and then, to the low affinity site. (2) The binding sites may have had slightly different conformations. Thus, the antibodies would bind first with the site for which they had a better fit, and then with the related one. (3) When some of the monoclonal antibodies bound to the antigenic site, they may have induced a conformational change in the antigen resulting in the exposure of a second epitope that was recognized by the remaining antibodies. (4) The two antibody combining sites of these monoclonal antibodies had different affinities for CPSMV I. At low concentrations (high dilutions) they bound to epitopes on the antigen for which they had a high affinity and at high concentrations (low dilutions) they bound to the site for which they had a low affinity. Both the consecutive and simultaneous assay curves of the following monoclonal antibodies were monophasic which suggests that they bound to one site: 5B, 2-1F, B12, 6A, 3-11C, 2-7A, 2-11A, F1, and H6 (Fig. 8 C, and E-L, respectively). The remaining monoclonal antibodies may have bound to two sites (4-5B, 3-9C, and 4-5F; Fig. 8 A, B, and D, respectively) since the simul-

taneous assay curve of monoclonal antibody 4-5B (Fig. 8A), both the consecutive and simultaneous assay curves of antibody 3-9C (Fig. 8B), and the consecutive assay curve of monoclonal antibody 4-5F (Fig. 8D) are all diphasic. Whether the cause of the diphasic curves is differing affinities of the antibodies for two sites, variable conformation of the two epitopes, appearance of a second site due to conformational changes in the antigen, or antibodies with combining sites of two different affinities for the antigen, cannot be determined from the information obtained in this study.

The intent of the competition assays was to characterize 17 monoclonal antibodies produced against CPSMV I and II to identify those which could be utilized, in future studies, to determine the topographical arrangement of epitopes of different serotypes of CPSMV I. The unlabelled antibodies were first categorized into those that did and those that did not compete with the the biotinylated antibody H6. The former, in turn, were classified according to the impact that antibody affinity and/or concentration had on ability to compete. The antibodies that did compete were also subdivided into those antibodies which competed for either one or for two sites on CPSMV I. When all of these characteristics were considered, the following suggestions can be made as to which

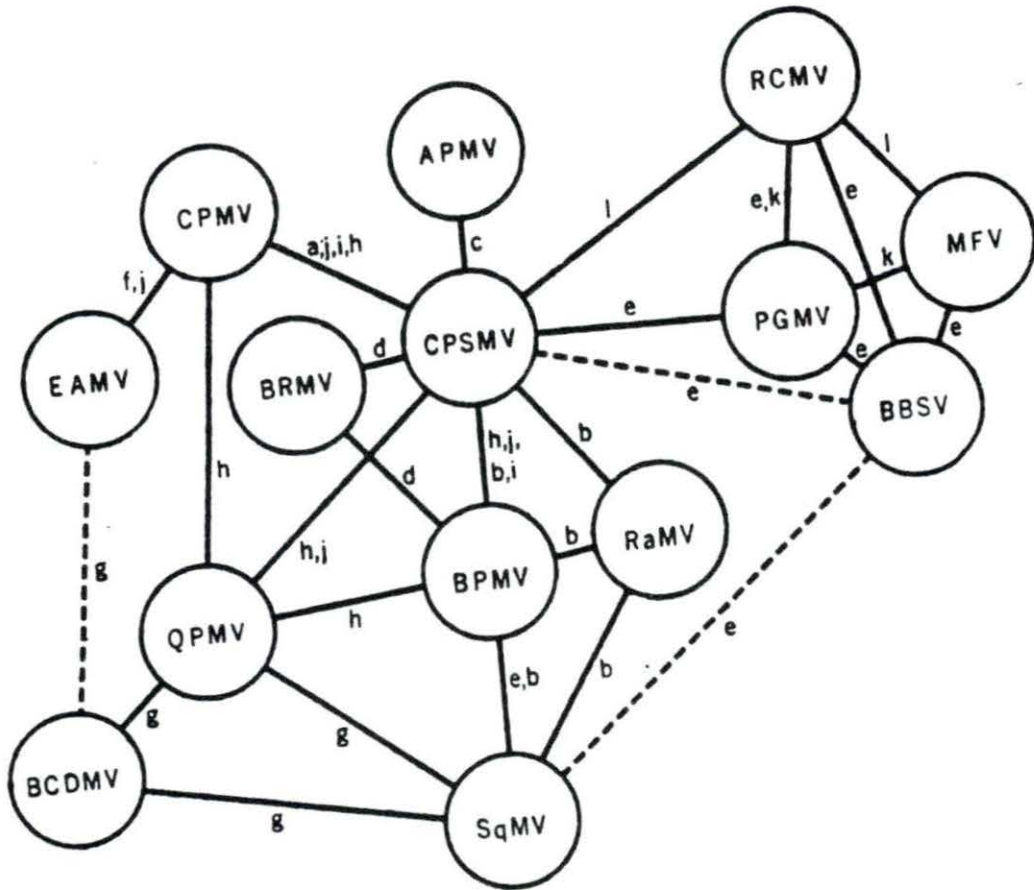
antibodies could be utilized to study the epitope topology of different strains of CPSMV. Monoclonal antibody H6 (Fig. 8L) should be utilized since it is the antibody homologous to the biotinylated antibody H6. Antibody 4-5B (Fig. 8A) could be included since it is the only antibody to combine the following characteristics: its concentration had a greater impact upon its ability to compete than its affinity, it is a low affinity antibody that was very rapidly displaced by the biotinylated antibody H6 (as indicated by the steepness of the slope of the consecutive assay), and it also appeared to bind to two sites on CPSMV I. One of the monoclonal antibodies 5B, 2-1F, B12, 6A, 2-11A, and F1 (Fig. 8 C, E-G, J and K) could be employed since, in addition to antibody concentration having a greater impact upon ability to compete than affinity, antibody affinity appears to be greater than that of antibody 4-5B (Fig 8A), and they only bind to one site on CPSMV I. Antibody 4-5F (Fig. 8D) could be utilized for the same reason as those just cited except that it apparently bound to two rather than one site. Monoclonal antibody 3-9C (Fig. 8B) could be included since both its affinity and concentration affected its ability to compete with the biotinylated antibody H6, and it also bound to two sites on CPSMV I. Antibody 3-11C (Fig. 8H) could be employed because its affinity had a greater impact than

concentration upon ability to compete. In summary, monoclonal antibodies H6 (Fig. 8L), 4-5B (Fig. 8A), one of 5B, 2-1F, B12, 6A, 2-7A, and F1 (Fig. 8 C, E-G, J and K), 4-5F (Fig. 8D), 3-9C (Fig. 8B), and 3-11C (Fig. 8H) could be employed in competition assays to determine the topographical arrangement of epitopes on different serotypes of CPSMV. This information could, in the future, be related to the phenotypic expression of virus infectivity in different hosts by the serotypes of CPSMV. Breeders could then utilize this information to develop resistant hybrids of cowpeas or in the study of virus-vector relationships.

Figure 9. Diagram of serological relationships among comoviruses.

Solid lines represent serological relationships in which the heterologous titer was 1/64th or more of the homologous titer or in which an immunodiffusion pattern was judged to show a precipitin line whose intensity for the heterologous reaction was not much less than that for the homologous reaction. Dashed lines represent serological relationships that meet these criteria barely or questionably. References: (a) Agrawal and Matt, 1964; (b) Cambell, 1964; (c) Fribourg et al., 1977; (d) Gamez, 1972; (e) Gibbs et al. 1968; (f) Jones and Barker, 1976; (g) Meiners et al., 1977; (h) Moore, 1973; (i) Shepherd, 1963; (j) D. J. Siler, H. A. Scott and G. Bruening, unpublished observations cited in Tomlinson, 1978; (k) Valenta et al., 1969; Valenta and Gressnerova, 1966; (l) Valenta and Marcinka, 1971





## SUMMARY

Monoclonal antibodies were produced against CPSMV serotype II. The cell lines producing the antibodies were selected by indirect ELISA which was also used for isotyping the antibodies. Selected antibodies of the IgM class were isolated with an anti-mouse IgM agarose affinity column.

Immunoblot analysis was utilized to characterize monoclonal and polyclonal antibodies and to determine the antigenic relationships of ten plant viruses, an animal virus and other unrelated antigens. Many of the antibodies employed in the assays cross-reacted with the viruses and other antigens. This may indicate that many non-specific protein-protein interactions took place, that some epitopes may be relatively ubiquitous among unrelated antigens, that the hybridoma cells produced antibodies with diverse specificities, and/or that the antibodies bound non-specifically due to the presence of calcium and magnesium in the dilution buffer used for the antibodies.

Electron microscopy suggested that CPSMV II and SMV do not share epitopes for the monoclonal antibody produced by cell line 4-5B. This confirms results obtained by immunoblot assays, i.e., antibody 4-5B reacted with CPSMV II but not with SMV.

Competition assays were used to characterize

monoclonal antibodies produced against CPSMV I and II since the immunoblot assays were equivocal in resolving this issue. This was accomplished by determining whether the antibodies competed against the biotinylated antibody H6, whether their affinity or concentration had a greater impact upon their ability to compete, and whether they competed for one or two binding sites on CPSMV I. Twelve of the 17 antibodies did compete, to varying degrees. Suggestions were made of antibodies that could be utilized, in the future, in assays to determine the topographical arrangement of epitopes on different serotypes of CPSMV.

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