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Relationships among serotypes of cowpea severe mosaic virus as determined by signature analysis

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Relationships among serotypes of cowpea severe mosaic virus as determined by signature analysis

by

Rong Di

A Thesis Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

Major: Plant Pathology

Signatures have been redacted for privacy
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The goal of this study was to determine the applicability of a technique, signature analysis, for examining the serological relationships among serotypes of a plant virus. Cowpea severe mosaic virus (CPSMV) was used as a model system for this study. Signature analysis is a relatively new serological approach which employs radioimmunoassay and monoclonal antibodies (MAbs) to examine the subtle antigenic differences among isolates of viruses. It has only been used to study animal viruses, e.g., hepatitis B virus (Wands et al., 1984) and dengue-2 virus (Monath et al., 1986). With this approach, far more antigenic heterogeneity has been demonstrated in these two viruses than previously shown by polyclonal antibodies.

CPSMV, a comovirus, includes nine serotypes (I to IX) determined by immunodiffusion tests with polyclonal antibodies (Lin et al., 1981, 1984, and Lin, personal communication, Bioplanta Tecnologia de Plantas S. A., Brazil). Several common or specific antigenic determinants have been identified. This makes CPSMV an ideal model to study whether signature analysis can be used to reveal the subtle antigenic differences among serotypes of a plant virus.

The theory of MAb was first developed in the late 1950s and achieved practical application in the late 1970s. Presently, MAbs are widely used in both animal and plant
sciences. For the purpose of this study, the most significant properties of MAbs are their greater specificity and homogeneity as compared to polyclonal antibodies. MAbs were made to CPSMV I, II and III. They were characterized by simultaneous and consecutive competition ELISAs (enzyme-linked immunosorbent assays) for their binding characteristics to serotypes of CPSMV.

Seven MAbs were selected to form a panel for use in biotin-avidin-based signature analysis assays to study the serological relationships among nine serotypes of CPSMV. With this study, signature analysis has been proven to be able to identify antigenic divergence among isolates of plant viruses.
LITERATURE REVIEW

Cowpea Severe Mosaic Virus

Cowpea severe mosaic virus (CPSMV, R/1:*/*:S/S:S/C, Ve/Cl) belongs to the comovirus group (de Jager, 1979). CPSMV, a multicomponent virus, consists of three icosahedral particles with a diameter of approximately 25 nm which can be separated into three centrifugal components: top (T), consisting of empty protein shells; and two nucleoproteins, middle (M) and bottom (B). The M and B particles are morphologically and serologically identical but contain RNA molecules of different molecular weight (Shepherd, 1964; Thongmeearkom and Goodman, 1978).

According to the original CMI/AAB (Commonwealth Mycological Institute of Association of Applied Biology) description of cowpea mosaic virus (CPMV), all isolates of CPMV could be separated into severe and yellow subgroups based on serological and host reactions (van Kammen, 1971). Later, Swaans and van Kammen (1973), after a thorough study of host range, symptomatology and the ratio of virus components of the two subgroups, concluded that they should be considered as two different viruses. For example, the symptoms of both subgroups differ markedly on most species tested; each isolate possesses a specific antigenic group and there is no homology in nucleotide sequence between the RNAs of the two isolates. Based upon serological differences, Fulton and Scott (1979)
proposed the serogrouping concepts for legume comoviruses and considered the severe and yellow subgroups as distinct viruses which were designated as the CPMV-Arkansas and CPMV-Sb serogroups, respectively. The recent CMI/AAB Description of Plant Viruses also considered the severe and yellow subgroups as distinct viruses and officially designated them as cowpea severe mosaic virus (CPSMV) and cowpea mosaic virus (CPMV), respectively (de Jager, 1979; van Kammen and de Jager, 1978).

The studies of Lin et al. (1981) showed that single-lesion isolates of cowpea severe mosaic virus obtained from central Brazil could be differentiated by immunodiffusion tests, in agar gel, into two serologically distinct groups; namely, serotype I and serotype II. Isolates of serotype I infected the soybean (Glycine max) cultivar IAC-2 which was immune to serotype II. Two additional serotypes III and IV were identified from CPSMV isolates (Lin et al., 1984). Only serotype III could infect Nicotiana tabacum "TNN". Serotype IV was unable to infect Chenopodium amaranticolor, a known diagnostic host for this virus. Two beetle species, Cerotoma arcuata and Diabrotica speciosa, could transmit serotype IV from beans to beans. Two major proteins with apparent molecular weights of 40,300-41,800 daltons and 20,000-21,400 daltons were detected in the middle components of the four serotypes. A minor protein of about 22,400-23,300 daltons was detected in serotypes I, III and IV, but not in serotype II.

Serological comparison, by immunodiffusion tests, showed
that the four serotypes had a common antigenic determinant designated "A". Serotypes I, III, and IV shared an additional common determinant designated as "B". Serotypes I and IV had the common determinant designated as "C". Each serotype also had a specific antigenic determinant designated as "D", "E", "F" or "G" for serotypes I, II, III, and IV, respectively.

Lin (personal communication, Bioplanta Tecnologia de Plantas S. A., Brazil), using the same immunodiffusion tests, identified five other serotypes of CPSMV; namely V (CR7), VI (S-1), VII (Ill), VIII (DG), and IX (ATCC-PV273).

CPSMV has several synonyms: Arkansas cowpea virus (Shepherd, 1964), Trinidad cowpea mosaic virus (Chant, 1962) and CPMV-severe subgroup (Thongmeearkom and Goodman, 1978).

Diseases caused by CPSMV are found in USA, Trinidad, Puerto Rico, El Salvador, Costa Rica, Venezuela, Surinam, Brazil, and Peru (de Jager, 1979). The disease incidence in cowpea (Vigna unguiculata) may be up to 100% (Dale, 1949;). Reduction of up to 50% in the number of pods and fresh weight was observed in CPSMV infection on cowpea (Debrot and de Bojas, 1967). The host range of CPSMV includes many leguminous crops and weeds and some solanaceous plants such as Phaseolus, Chenopodium amaranticolor and Nicotiana benthamiana (Anjos and Lin, 1984; Fribourg and Koenig, 1985). Almost all host plants develop necrotic or chlorotic lesions on inoculated leaves. Systemic symptoms are mottle or mosaic which often includes severe blistering and distortion of

CPSMV is mechanically transmissible (de Jager, 1979). It is also transmitted by leaf-feeding beetles. Those of the family Chrysomelidae, Cerotoma ruficornis and C. trifurcata are among the most important (de Jager, 1979). A seed transmission rate of 8% was reported for the Trinidad isolate in asparagus bean (*Vigna sesquipedalis*) (Dale, 1949).

Amorphous inclusion bodies were found near or surrounding the nucleus in epidermal strips of infected cowpeas and peas (Agrawal, 1964; Swaans 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 from $10^{-4}$ to $10^{-5}$ and a longevity *in vitro* of one to five days at a temperature of 24°C.

Monoclonal Antibodies

Immunoassays have been used by scientists working in all areas of biology. Antibodies can be generated that are specific for substances to be studied, and such antibodies can be employed in any of many standard immunoassays. However, many scientists are frustrated by the unpredictability of the immune response (Scharff, 1984). Some macromolecules are only weakly immunogenic, and it may be difficult to obtain high titer antisera for their detection. At least 100 µg of antigen, in relatively pure form, is required even for
substances that can induce an excellent antibody response. If impure antigens are used, the antiserum must be extensively cross-absorbed with heterologous antigens to achieve strain-specificity. Even if large amounts of a pure and highly immunogenic immunogen are injected, the recipient animal will make antibodies that differ widely in affinity and ability to induce precipitation, fixation of complement and binding to phagocytic cells. Each bleeding must be considered as a unique serological reagent and the amount of antibody obtained is often inadequate. These, and other problems, have led immunologists to seek techniques for producing more homologous and reproducible immunoreagents in larger amounts.

The theory of MAb developed from the hypothesis of clonal selection (Burnet, 1959). It assumes that, in animals, there exist clones of mesenchymal cell which carry immunologically reactive sites corresponding, in appropriate complementary fashion, to one potential antigenic determinant. When an antigen is introduced it will make contact with a cell of the corresponding clone which is presumably a lymphocyte. By this mechanism, production of globulin molecules of the characteristic type is stimulated.

The achievements of: 1) in vitro culture of normal and malignant cells, 2) chemically defined culture media, and 3) usage of Sendai virus and polyethylene glycol-based fusing technology, made the development of hybridoma technology possible (Goldsby, Srikumaran and Guidry, 1984). Cohn (1967)
and Potter (1972) found that tumors induced by injecting mineral oil into the peritoneal cavity of mice could be passed to other mice and a continuous supply of antibodies of a single specificity could be achieved. In 1973, Schwaber and Cohen used inactivated Sendai virus to fuse human lymphocytes from normal donors to a mouse plasmacytoma. They were the first to demonstrate that one could immortalize the antibody production of a normal cell of the B lymphocyte lineage by fusing it in vitro with a plasma cell tumor.

In 1975, Kohler and Milstein devised and demonstrated a strategy for the deliberate and rational construction of continuous cell lines that secreted MAb of desired specificity. They fused HAT (hypoxanthine, aminopterin and thymidine)-selectable mouse myeloma cells with spleen cells from mice that had been previously immunized with sheep red blood cells. They then screened the resulting hybrids for the production of antibody specific for sheep red blood cells.

MAb can provide several advantages as compared with polyclonal antibodies. Ten times less antigen are required for immunization. MAbs have greater specificity than polyclonal antibody. The molecular homogeneity of a MAb ensures that only one antigenic determinant of the antigen is analyzed at one time. Therefore, it is possible to demonstrate fine distinctions between closely related virus strains in a reproducible manner. Additionally, once a useful hybridoma has been established, virtually unlimited quantities
of a particular MAb can be produced.

Conversely, MAbs also have disadvantages. Generating MAbs is very time-consuming, expensive and it requires a high level of expertise. Difficulties may exist in generating MAbs against weakly immunogenic molecules. This can also be true for generating polyclonal antibodies (Scharff, 1984). The very high specificity of MAbs may also be a problem for some applications, as they may recognize only the immunizing serotype of the antigen and not other prevalent serotypes. In addition, Van Regenmortel (1984) reported that some of the MoAbs specific to a particular strain of TMV often reacted better with other strains. He suggested that heterospecificity is more obvious with MAbs than with polyclonal antibodies because the heterospecificity is not masked by the other antibodies found in an antiserum. The selection of MAbs specific for common epitopes of different serotypes as well as the judicious mixture of different MAbs should make it possible to overcome these difficulties. Diaco et al. (1986) reported that some MAb preparations produced against barley yellow dwarf virus (BYDV), although of equivalent titre, were more reactive with heterologous than with homologous isolates of BYDV. This phenomenon has been referred as heterooclatic, or heterospecific (Al-Moudallal et al., 1982).

The hybridoma technique has been used extensively in medical and veterinary studies. Only since the early 1980s,
has it been introduced into the research of plant pathology. MAbs specific for more than 20 plant viruses have been obtained (Van Regenmortel, 1984). The detection of antigenic changes induced by a single amino acid substitution in the protein coat of tobacco mosaic virus has been achieved by MAbs (Al Moudallal et al., 1982). Hill et al. (1984) demonstrated that it was possible to detect soybean mosaic virus, lettuce mosaic virus and maize dwarf mosaic virus successfully, using a single MAb in competitive radioimmunoassays. Due to the unique specificity of MAbs, they have proven to be valuable reagents for virus classification and serotyping of viruses (Halk et al., 1984).

**Immuoassay**

Various immunoassays have been applied for the detection and grouping of plant viruses. For example, immunodiffusion tests have provided strong evidence for existence of serotypes of CPSMV (Lin et al., 1984). Many of these tests are easy to perform, but the results may take days to obtain. Immunoelectrophoresis tests have the advantage of being rapid. Immunofluorescence is rather subjective and is labor intensive; in contrast, radioimmunoassay (RIA) is objective and can be automated, but it often utilizes reagents with a rather short shelf life (Voller et al., 1980). In addition, researchers are subject to exposure to radioactive isotopes. Enzyme-immunoassay is also objective and can be automated; it
is free from the potential danger of radioactive substances and the reagents used have a long shelf life. Only cheap simple equipment is required. In addition, since both ELISA and RIA are governed by the same thermodynamic constraints, and the enzyme can be detected in the same range of molarity as commonly used radioisotopes, the sensitivity and specificity of RIA and ELISA are comparable (Berzofsky and Berkower, 1984).

For the detection of plant viruses, the "double antibody sandwich" form of ELISA has been found to be suitable (Voller et al., 1976). In this method, the specific antibody has been first adsorbed onto a solid surface to selectively trap and immobilize the virus in the test sample. A second enzyme-linked antibody is allowed to react with the trapped virus. This complex of enzyme-labeled antibody with the antigen is then detected colorimetrically by adding an appropriate suitable enzyme substrate. In general, this system can detect viruses at concentrations of 10-100 ng/ml (Voller et al., 1976). However, Clark and Adams (1977) have reported detection levels as low as 1 ng/ml.

Variants of ELISA were compared by Koenig and Paul (1982) for the detection and differentiation of plant viruses. Indirect ELISA on plates not precoated with antibodies was found to detect the broadest range of serologically related viruses. In this assay, the antigen to be tested is bound directly to the solid surface, followed by a specific
antibody. An enzyme-labelled antiglobulin, reactive with the specific antibody is then added. After adding the enzyme substrate, the color change is measured spectrophotometrically. In this assay, commercially available enzyme-labeled antiglobulin can be used. This obviates the storage and preparation of many different conjugated antibodies.

Indirect ELISA can also be utilized in the screening process to identify hybridomas which produce specific MAb. It is a highly sensitive screening test for hybrid cell cultures at early stages of their development. In addition, it can be rapidly performed on a large number of samples. It also has the advantage of being independent of the hybridoma antibody isotype (Yewdell and Gerhard, 1981). Media from hybridoma cells growing in tissue culture plates are transferred to an ELISA plate which has been coated with antigen and blocked with a blocking agent. Enzyme-conjugated rabbit or goat anti-mouse IgG or IgM and enzyme substrate are added, and the reaction products are measured spectrophotometrically. In this manner, wells in the tissue culture plate which contain specific antibody-secreting hybridomas can be identified.

The biotin-avidin system is another alternative for enzyme immunoassays. It was originally described by Bayer and Wilchek (1974) for immunochemical staining. Avidin, a glycoprotein with a molecular weight of 67,000, has an exceptionally high affinity for biotin (dissociation constant:
$10^{-15}$ M) (Green, 1963). It possesses 4 active sites for binding biotin. In addition, the biotin-avidin system offers several other advantages: (1) both biotin and avidin are stable; (2) biotin can be attached to small ligands and macromolecules efficiently and under very mild conditions; (3) usually, the physical characteristics and biological activity of biotin-derivatized proteins are not changed; (4) both biotin and avidin are relatively inexpensive (Bayer et al., 1979).

Guesdon et al. (1979) introduced the biotin-avidin system into enzyme-immunoassays. It was found that the antigen-binding capacity of antibody was not modified even with extensive substitution by biotin for amino groups in antibody molecules. Kendall et al. (1983) reported that an ELISA procedure involving biotinylated goat anti-mouse gamma immunoglobulin (IgG) and avidin-alkaline phosphatase was 4134 times more sensitive than AUSAB (a commercial radioimmunoassay, Abbott Laboratories, North Chicago, IL) for the detection of mouse antibody to hepatitis B surface antigen. Diaco et al. (1985) used a biotinylated second antibody and an avidin-alkaline phosphatase detection system in a double-antibody sandwich ELISA to detect soybean mosaic virus (SMV) in soybean seed extracts. It was found that this system could detect less than 10 ng of SMV/ml, which was more sensitive than 25 ng/ml detected by a polyclonal antibody-based solid-phase radioimmunoassay (SPRIA). Furthermore, the
biotin-avidin system required much less time for seed sample assays than SPRIA did.

Topographic Analysis

The discriminatory power of MAbs makes them an ideal reagent for investigation of antigenic relationships between viral proteins. They are invaluable for identifying minor antigenic differences between closely related proteins. There are three types of experiments that can provide insight into the antigenic topology of viral proteins (Yewdell and Gerhard, 1981). The first is to construct an operational antigenic map by comparative antigenic analysis of antigenic mutant proteins. The second type of experimental approach is to study the capacity of antibodies to compete for antigenic sites on the viral protein. Results are then correlated with the functional and physical antigenic topology of the virus. The examination of the structural properties of the epitopes themselves is the third type of experiment.

In an antibody competition study, one MAb carries a marker, and unlabeled antibodies are examined for their ability to inhibit binding of labeled antibody with antigen. The antibody can be labeled with radioisotope or with biotin as described previously. If the unlabeled antibody fails to reduce the binding of labeled antibody, the two antibodies bind to distinct epitopes on the antigen. If antibodies are observed to compete for binding to antigen, several
explanations are possible. First, the antibodies may delineate structurally overlapping epitopes. Second, binding of one antibody may prevent access of the second antibody to a physically distinct site; in this case the proximity of the two epitopes creates steric hindrance between the antibody molecules. Third, the binding of some MAbs may induce conformational changes in the antigen and thereby alter the epitope recognized by the competing antibody. There is also another possibility, suggested by Friguet et al. (1983), that the antigen oscillates between two conformations; if the first antibody species binds exclusively to one conformation while the second one binds exclusively to the other, it will be impossible for the two antibodies to bind simultaneously to the antigen even if they are specific for distinct epitopes. Antibody competition assays tend to exaggerate the physical proximity between epitopes. Thus, competition assays provide the most information when the binding of antibodies to a protein can be shown to be noncompetitive.

Both simultaneous and consecutive (sequential) competition assays can be used to characterize competition between antibodies (Yewdell and Gerhard, 1981). In the former assay, the unlabeled antibody and the labeled antibody are added together. If the binding of the labeled antibody is decreased by 50% or more, true competition is likely. If binding of the labeled antibody to the epitope is decreased by 100% in the consecutive competition assay, true competition is
likely (Kubanek, 1987). As suggested by Schmaljohn et al. (1983), if the unlabeled antibody caused at least a 50% inhibition in binding of the labeled antibody, the two antibodies were presumed to bind to the same or to the topographically related epitopes.

The antibody competition assays delineate and characterize the antigenic topographic map of antigens. Breschkin et al. (1981) studied the topography of antigenic determinants of influenza virus hemagglutinin by competitive RIA. The unlabeled MAb was used to block the binding of radiolabeled heterologous MAbs. Pairs of MAbs that competed completely were considered as binding in the same antigenic region. Other pairs bound non-competitively to physically distinct regions. Thus, the existence of four distinct antigenic sites was demonstrated. Sindbis virus epitopes were grouped into six spatially distinct antigenic sites by Schmaljohn et al. (1983). Employing ELISA, the binding of individual peroxidase-labeled MAbs to immobilized (solid-phase), detergent-disrupted Sindbis virus was inhibited specifically by one or more unlabeled antibodies.

These techniques, delineating antigenic topographic maps of antigens, have been used in plant virology. The studies by Dougherty et al. (1985) indicated that MAbs to tobacco etch virus (TEV) capsid protein could be grouped into two classes. One group recognized surface epitopes specific for TEV; the other one defined internal epitopes which were not unique to
TEV. Diaco et al. (1985) demonstrated that there were at least two epitopes present on the coat protein of the P-PAV isolate of BYDV in a competition ELISA. In this system, one MAb was labeled with biotin; its binding to the antigen was inhibited by an unlabeled antibody.

Signature analysis is a relatively new technique which has been used in animal virology (Wands et al., 1984; Monath et al., 1986). This approach employs radioimmunoassay and a small panel of labeled MAbs specific for different epitopes (Van Deusen, 1984). When comparing subtypes of the virus, differences in the relative occurrence of each epitope as well as variations within the epitopes are observed. This method avoids the necessity for the knowledge of viral protein concentration in complex protein mixtures in order to identify differences in antigens.

In signature analysis, a group-reactive MAb with high avidity is used to capture virus onto the solid phase. The solid phase is incubated with dilutions of antigen and iodinated antibody. The bound radioactivity is counted and graphed against the antigen concentration. Thus, the "signature" or "fingerprint" of the immunoreactivity between antigen and the iodinated antibody is generated.

Using this approach, Wands et al. (1984) showed that hepatitis B (HBs) viral antigen exhibited far more antigenic heterogeneity than previously recognized by polyvalent anti-HBs antibodies; i.e., there were subtypes within the classic
HBs antigen subtypes. Also by signature analysis, Monath et al. (1986) revealed striking antigenic differences among the prototype and four geographic topotype strains of Dengue-2 virus, and a high degree of antigenic similarity among strains from the same geographic region.
MATERIALS AND METHODS

Distilled and Deionized Water

Distilled and deionized water (ddH\textsubscript{2}O) was used in the preparation of all media and buffers. This was obtained by passing distilled water through a Super-Q Ultra Pure Water System (Millipore Corp., Bedford, MA), which removed both organic and inorganic ions.

Virus Propagation

Cowpea severe mosaic virus (CPSMV) serotypes I to IX were propagated in and purified from \textit{Vigna unguiculata} Walp. (California blackeye cowpea) (Lin et al., 1981). Approximately 0.2 g of desiccated, infected leaf tissue was ground in 2 ml of 0.05 M phosphate buffer (sodium, potassium phosphate, pH 7.0) and used to mechanically inoculate leaves of 5 cowpea plants which had been dusted with 600 mesh carborundum. Fourteen to twenty days later, systemically infected leaves were collected and ground in 0.05 M phosphate buffer and used to inoculate a large number of cowpea plants for virus propagation.

Purification of Cowpea Severe Mosaic Virus

The purification procedure described by Lin et al. (1981) was modified and used for isolating CPSMV.

Fourteen days after inoculation, infected cowpea leaves
were harvested, weighed and homogenized in 2 volumes of borate buffer (0.1 M boric acid, 0.01 M ethylenediamine-tetraacetic acid, 0.1% sodium sulfite, pH 7.6) with a Waring blender. The homogenate was filtered through two layers of cheesecloth. Butanol was added to a final concentration of 8% (v/v) to the filtrate. The mixture was incubated at 22 C for 5 minutes and then centrifuged at 11,700 x g for 20 minutes. The supernatant was strained through glass wool to remove large pieces of plant debris. To precipitate the virus, PEG (polyethylene glycol, MW 6000) and NaCl were added to final concentrations of 6% (w/v) and 0.01 M, respectively. The mixture was stirred for 20 to 30 minutes and then stored at 4 C for 2 hours. The precipitated virus was collected by centrifugation at 11,700 x g for 25 minutes. The pellet was covered with 0.05 M phosphate buffer and placed on a shaker overnight at 4 C.

Further purification of the virus was achieved by 2 to 3 cycles of differential centrifugation. The resuspended mixture was centrifuged at 8,700 x g for 10 minutes. The virus was concentrated from the supernatant by centrifugation at 77,600 x g for 2 hours and resuspended in 0.05 M phosphate buffer.

The resuspended virus was centrifuged through a linear sucrose gradient [10%, 20%, 30%, 40% sucrose (w/v) solution in 0.05 M phosphate buffer] using a Beckman SW27 rotor at 65,000 x g for 1.5 hours. Virus was collected with a density
gradient fractionator (Model 640; ISCO, Lincoln, NE) equipped with a monitor at 254 nm. The fractionated virus was pooled and dialyzed overnight against 0.05 M phosphate buffer. Finally, virus was concentrated by centrifuging at 77,600 x g for 2 hours and resuspended in 0.05 M phosphate buffer. Virus concentration was measured spectrophotometrically by using \( E_{280}^{0.1\%} = 8.0 \) (van Kammen, 1971).

Production of Hybridomas

**Immunization of mice**

BALB/c mice (Jackson Laboratory, BarHarbor, ME) were used to produce hybridomas. Fifty Ig purified virus (CPSMV III) in 0.1 ml 0.05 M phosphate buffer was emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO; #F-5881). Female mice were used and injected with virus intraperitoneally. One month later, at an interval of 3 days, the mice were twice boosted intravenously through the tail vein with the same amount of virus. Three days after the second hyperimmunization, a mouse was exsanguinated and the serum was retained as a positive control for screening assays.

**Media and chemicals used in hybridoma production**

Dulbecco's modified eagle medium (DMEM) was the basic nutrient medium which consisted of:

DMEM (Gibco Laboratories, Grand Island, NY; #430-2100) 5 L package
N-2-Hydroxyethylpiperazine-N-2-
ethanesulfonic acid (HEPES; Sigma #H-3375) 35 g
NaHCO₃ 18.5 g
ddH₂O up to 5 L

The medium was filtered through a 0.4 um Zetapor filter (AMF Cuno, Meriden, CT; #NM142-01-020sp) and then through a 0.2 um Acro 50A filter (Allied Fisher Scientific, Itasca, IL; #09-730-241G). The medium was collected and stored at 4 C.

Regular growth and maintenance medium (DMEM-R) DMEM-R was used for the growth, maintenance and revitalization of hybridoma cells. It was prepared by adding 1 ml L-glutamine solution (0.2 M in H₂O), 7.5 to 10 ml of horse-calf serum (2 parts horse to 1 part calf serum; Gibco Laborotories, Grand Island, NY; #230-6050 and #230-6170) into DMEM to a final volume of 100 ml. It was filtered through a 0.2 um cellulose acetate syringe filter (Allied Fisher Scientific #09-740-35A).

Myeloma cells The Sp2/0-Ag14 (Sp2/0) myeloma cells were originally provided by Dr. R. A. Van Deusen (National Veterinary Services Laboratories, Ames, IA). Cells were maintained by transferring to DMEM-R three times a week.

8-Azaguanine (8-aza) medium 8-aza medium was used to eliminate Sp2/0 mutants resistant to aminopterin which arise during the regular growth period. 8-aza solution (1X) was prepared by adding 10 ml ddH₂O to the powder to make a final concentration of 6.6 x 10⁻³ M (Sigma #A-8256). 8-aza medium
was DMEM-R medium containing 1% 8-aza solution (1X). Every three months, Sp2/0 cells were passaged through 8-aza medium twice.

**Conditioned medium** Sp2/0 cells were grown in DMEM-R for 3 days until the medium became orange-yellow. Cells were concentrated by centrifuging at 2000 rpm for 10 minutes, and the conditioned medium was filtered through a 0.2 μm syringe filter to eliminate remaining cells.

**HAT medium** Hypoxanthine, aminopterin and thymidine were used to select spleen-Sp2/0 hybrid cells (Yelton and Scharff, 1981). The Sp2/0 myeloma cells lacked the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT). They could not use exogenous hypoxanthine to synthesize purines, and died in the presence of aminopterin, which blocked de novo synthesis of purines and pyrimidines. Spleen cells, although not killed by aminopterin, could not be cultured indefinitely. Therefore, after a few days the only rapidly dividing cells remaining were Sp2/0-spleen hybrids. HAT medium consisted of:

**HAT solution (100X)** (Hazelton-Dutchland Inc., Denver, PA; #59-77076)

- Conditioned medium: 1 ml
- Horse-calf serum: 50 ml
- DMEM: 10 ml
- L-glutamine solution: 38 ml

**HT medium** It was made in the same manner as HAT
medium except that hypoxanthine-thymidine (HT; Hazleton-Dutchland Inc. #59-57076) was substituted for HAT. HT medium was a transition medium for hybridomas from HAT medium to DMEM-R medium.

**Phosphate buffered saline (0.01 M PBS, pH 7.2)**

Dibasic sodium phosphate (0.01 M) containing 0.85% NaCl was prepared and titrated to pH 7.2 with 0.01 M monobasic sodium phosphate containing 0.85% NaCl.

**Polyethylene glycol (PEG)**

PEG was utilized to break or weaken cell membranes and enhance fusion of cells. PEG solution was prepared by adding 2.7 ml of 0.01 M PBS to 2.25 g PEG (MW 1000; Hazleton-Dutchland Inc. #59-90739) and dissolving in a 60°C water bath.

**Freezing medium**

This medium was made by adding 10 ml dimethyl sulfoxide (DMSO; Sigma #D-2650) to 90 ml of sterile horse-calf serum.

**Production of hybridomas**

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

Three days prior to cell fusion, Sp2/0 cells were passed in DMEM-R medium and the mouse was hyperimmunized for the second time. After exsanguination, the spleen was removed aseptically. The spleen was rinsed with 5 ml DMEM in the first sterile 60 mm plastic petri dish and then transferred to
the second petri dish. It was perfused with 5 ml DMEM by using a 2 ml syringe. Over the third petri dish, the spleen tissue was macerated through a 100 mesh stainless steel screen and separated into single cells. The medium in the second petri dish was used to wash the screen. The spleen cells were mixed with Sp2/0 cells at a 1:1 ratio, and were centrifuged at 200 x g for 8 minutes. One milliliter of sterile PEG solution was added to the pellet slowly over a period of 15 seconds. The cells were gently mixed for 30 seconds and then incubated in a 37 C water bath for 90 seconds. One milliliter of DMEM was slowly added to the tube over a period of one minute and the tube was rotated gently to mix the cells at the same time. After the addition of 10 ml more DMEM, the tube was incubated in the 37 C water bath for 5 minutes. The cells were collected by centrifuging at 200 x g for 8 minutes. The pellet was dispersed in 1 ml HAT medium and then resuspended to a concentration of 5 x 10^5 myeloma cells/ml with additional HAT medium. The cell suspension was plated at 0.2 ml/well in 96-well tissue culture plates (Corning Glass Works, Corning, NY; #25860). They were incubated at 37 C in an incubator enriched with 5% CO_2, (Forma Scientific, Marietta, OH; water-jacketed incubator).

Four days after fusion, old media in the wells were aspirated and new HAT medium was added. Three days later, cells were fed with HT medium. Another three days later, they were fed with HT medium and thereafter with DMEM-R at 2 to 3
day intervals.

When the bottom of the majority of the wells were one-half to two-thirds covered with hybridoma cells, the media were screened for antibody-producing cells by indirect ELISA.

Indirect Enzyme-Linked Immunosorbent Assay

A procedure similar to that described by Voller et al. (1976) was used. The buffers used in indirect ELISA were prepared in the following manner:

**Virus coating buffer**  This 0.05 M sodium-potassium-phosphate buffer (pH 7.0) was the same as the one used in virus purification.

**Wash buffer (PBS-Tween, 20X)**

- **NaCl**: 160.0 g
- **KH₂PO₄**: 4.0 g
- **Na₂HPO₄**: 23.8 g
- **KCl**: 4.0 g
- **Polyoxyethylenesorbitan monolaurate** (Tween 20; Sigma #P-1379): 10.0 ml
- **Sodium azide**: 1.0 g
- **dd H₂O to a final volume of**: 1000 ml

ELISA plates were washed with 1X wash buffer between steps to reduce non-specific binding. Wash buffer was also used to prepare blocking buffer and to dilute antibodies.

**Blocking buffer (5% blotto)**  The excess sites after
binding of antigen to the well were blocked by blocking buffer to reduce the non-specific binding.

Non-fat dry milk (Carnation brand)  5 g
Antifoam A emulsion (Sigma #A-5758)  33.3 ul
1X wash buffer to  100 ml

After stirring for 30 minutes, the mixture was filtered through Whatman #1 filter paper, and the pH was adjusted to 7.4.

**Substrate buffer**

- Diethanolamine  10.0 ml
- MgCl$_2$·6H$_2$O  10.0 mg
- Sodium azide  25 mg
- dd H$_2$O to  100 ml

HCl (1 N) was used to adjust the pH to 9.8.

One phosphatase substrate tablet (P-nitrophenyl phosphate, disodium, 5 mg/tablet; Sigma #104-105) was dissolved in 5 ml substrate buffer to prepare the substrate solution.

At a concentration of 0.5 ug/ml in coating buffer, 50 ul CPSMV III was added to each well of a polystyrene Immulon I plate (Dynatech Laboratories, Alexandria, VA). After incubation at 37 C for 1 hour, the plate was washed with wash buffer three times for 3-5 minutes, and then blocked with 300 ul of 5% blotto. The plate was incubated at 37 C for 1 hour and washed afterwards. Supernatants (50 ul) from the wells of tissue culture plates were added and incubated at 37 C for 1
hour and washed. Fifty microliter of alkaline phosphatase conjugated with rabbit anti-mouse IgG or goat anti-mouse IgM antibodies (Sigma #A-7784 and #A-1902, 1:1000 diluted in wash buffer), were added to each well. The plate was incubated at 37 C for 1 hour and washed. After 50 ul substrate solution was added, the plate was incubated at 37 C for 30 to 60 minutes or until a yellow color change was observed. Reactions were stopped by adding 50 ul of 3 N NaOH to each well. The reaction product could be observed visually for color change from colorless to yellow, or it could be measured photometrically at 410 nm with a Dynatech Minireader II (Dynatech Laboratories). Hyperimmune serum was used as a positive control and conditioned medium as a negative control. The cells in the positive wells were saved for cloning.

Cloning

Two kinds of cloning procedures were used in this study. The first procedure (limiting dilution) was as described by Mernaugh et al. (1987). The hybridoma cells growing in tissue culture plates selected by indirect ELISA were resuspended and one drop of the cell suspension was transferred to 5.0 ml of DMEM-R (10^{-2} dilution). One drop of this mixture was transferred to the second 5 ml DMEM-R and mixed (10^{-4} dilution). Again, another 0.5 ml of cell suspension was added to 4.5 ml DMEM-R in the third tube (10^{-5} dilution). The second and the third cell suspension were plated at 0.2
ml/well into 96-well tissue culture plates.

The second procedure (calculation method) was as follows. After the hybridoma cells were counted, they were diluted in 20 ml DMEM-R to a final concentration of 5 cells/ml. Two-tenths milliliter of cell suspension (1 cell/0.2 ml) were added to each well of a 96-well tissue culture plate.

The plate was incubated in the 37 °C incubator enriched with 5% CO₂ and high humidity. Wells containing single cell colonies were selected under a light microscope. When the bottom of wells was two-thirds covered with cells, the culture medium was tested by indirect ELISA for the production of relevant MAbs. The positive clones were expanded in DMEM-R medium. After the immunoglobulin class and subclass of MAbs were determined, cells were frozen or injected into mice to produce ascitic fluid.

Isotyping of Monoclonal Antibody

A Streptavidin-Biotin System/MonoAb-ID EIA Mouse Kit (Zymed Lab. INC., San Francisco, CA) was utilized to determine the immunoglobulin class and subclass in a modified indirect ELISA.

The ELISA plate was coated with virus antigen, blocked with 5% blotto, and MAb in DMEM-R medium was added as in the indirect ELISA. Fifty microliter of biotinylated normal rabbit immunoglobulin (as negative control) or rabbit anti-mouse IgM (immunoglobulin M), IgG, IgG2a, IgG2b, IgG3 and IgA
(immunoglobulin A) were added to each well. The plate was incubated at 37°C for 30 minutes and washed with wash buffer. Peroxidase-labeled streptavidin (50 ul/well) was added to all wells. After incubation at 22°C for 30 minutes and washing, 100 ul of ABTS solution [2, 2-azino-di (3-ethyl benzthiazoline sulfonic acid)] was added and incubated at 22°C. The reaction products were determined with a spectrophotometer at 410 nm.

Freezing Cells

Hybridoma cells were centrifuged at 200 x g for 8 min and resuspended in 2 ml DMSO medium. Cells were transferred into an autoclaved Nunc tube (Nunc-Vangard International; Neptune, NJ). Nunc tubes were put into a grey Nunc mainer which was fitted inside a styrofoam freezing chamber. They were designed to let the temperature drop by 1°C/min until -100°C was achieved. They were placed in a -100°C cryogenic freezer. The second day, the tubes were removed from the styrofoam chamber and the Nunc mainer and stored at -100°C.

To revitalize the frozen cells, the Nunc tube was warmed rapidly between the palms of hands. The contents were centrifuged at 200 x g for 6 minutes. Cells were washed once with DMEM-R medium by centrifugation, resuspended in DMEM-R and plated in 96-well plate.
Production of Monoclonal Antibody

Flask media and mouse ascitic fluid were used as a source of MAbs.

To prepare flask media, cells were grown in DMEM-R medium for 14 to 20 days. The flask media were centrifuged at 1000 x g for 10 minutes, and the supernatants were stored at -70 C.

Mice were utilized to produce ascitic fluid which contained a higher concentration of specific MAb than did flask media. BALB/c mice were primed with 0.5 ml tetramethyl pentadecane (pristane; Sigma T-7640) one month prior to the injection of cells. One half milliliter of actively growing cloned cells (approximately 1 X 10^6 cells/ml) were centrifuged, resuspended in 0.5 ml of PBS, and were injected into the peritoneal cavity of the recipient mouse. Ten to fifteen days later, when the peritoneal swelling became pronounced, ascitic fluid was collected at 2-to 3-day intervals by inserting an 18 G needle into the peritoneal cavity. The ascitic fluid was clarified by centrifugation at 1000 x g for 10 minutes and stored at -20 C.

Purification of Monoclonal Antibody

Purification of IgM through a sepharose column

Monoclonal IgM was purified from ascitic fluid using the procedure described by Jones et al. (1988). Ascitic fluid was added dropwise to 0.32 M boric acid with gentle stirring (ascitic fluid:boric acid = 1:20, v/v). After incubation at
22 C for 30 minutes, the precipitated antibody was centrifuged at 800 x g for 10 minutes. The pellet was resuspended in 0.1 M Tris-HCl, pH 8.0, containing 0.15 M NaCl (0.1 M Tris buffer), and dialyzed against the same buffer overnight. The mixture was chromatographed on a Sepharose CL-6B gel filtration column (Pharmacia, Inc., Piscataway, NJ; 58 cm X 1.5 cm, Vo = 35.0 ml), using 0.1 M Tris buffer as the elution buffer. IgM, as the largest protein molecule, emerged in the first peak. One milliliter fractions, after the void volume, were collected and assayed by indirect ELISA for the presence of specific IgM. Protein concentration of MAb was estimated by using $E_{260}^{0.1\%} = 1.4$.

Purification of IgM through anti-IgM affinity column

Alternatively, another procedure was used to purify IgM from ascitic fluid. The chromatography column was constructed by using a 10 ml syringe which contained 5 ml wet volume of anti-mouse IgM (u-chain specific) agarose (0.4 mg of mouse IgM could be bound and eluted per milliliter resin; Sigma #A4540). The column was washed with 0.01 M PBS (pH 7.4). The ascitic fluid was adjusted to pH 7.4 and applied to the column. After washing with 20 ml of pH 7.4 PBS (0.01 M) and 10 ml pH 4.0 PBS (0.01 M), the bound IgM was eluted from the column in the second peak by washing with an appropriate amount of pH 3.0 PBS (0.01 M). The fractions were adjusted to pH 7.0 with 0.01 M NaOH and tested with an indirect ELISA for the presence of
IgM. The fractions that contained IgM were pooled and dialyzed overnight against 0.01 M PBS (pH 7.0).

**Biotinylation of Monoclonal Antibody**

The purified antibody was biotinylated by the method of Bayer et al. (1979). N-hydroxysuccinimidobiotin (biotin; Sigma #H-1759) dissolved in dimethylformamide (DMF) was added to monoclonal IgM in a 1:50 v/v and 20:1 mol/mol ratio. The mixture was rotated at 22 C for 4 hours, and was dialyzed at 4 C against 0.01 M PBS (pH 7.0) overnight. The concentration of biotinylated MAb was measured spectrophotometrically at $E_{280}^{0.1\%} = 1.4$. It was stored in 1 ml aliquots at -20 C.

**Competition Enzyme-Linked Immunosorbent Assay**

**Optimization for competition ELISA**

Indirect ELISA was used to optimize the concentrations of coating antigen, biotinylated antibody and avidin-labeled alkaline phosphatase. The plates were incubated at 37 C for 1 hour at each step and then washed with wash buffer in all the competition ELISA assays. The optimized conditions were achieved by calculation of P/N ratios (Hill et al., 1981) to yield the highest response (P) over background (N). The optimization procedures were as follows.

To optimize the concentration of coating antigen, purified virus was added to an ELISA plate at concentrations of 0.25, 0.5, 2, 5, 8, 10, 15, 20, 25 and 30 ug/ml (6 wells
per concentration). After the plate was blocked, 5 ug/ml (50 ul/well) biotinylated antibody was added to each of 3 wells at each antigen concentration; wash buffer was added to the other 3 wells as a control. Avidin-labeled alkaline phosphatase (2 ug/ml) and substrate were added stepwise. The P/N ratios were calculated as the ratio of the average absorbance in the presence of biotinylated antibody to that in the absence of biotinylated antibody. The concentration that had the highest P/N ratio was considered to be the optimum concentration.

To optimize the concentration of biotinylated antibody, antigen at the optimum concentration was applied to half of the wells in an Immulon I ELISA plate (50 ul/well), 0.05 M sodium-potassium phosphate buffer (pH 7.0) was added to the other half of the wells as a control. After blocking the plate, 50 ul/well of biotinylated antibody was added at concentrations of 0.5, 1, 2, 3, 4, 5, 6 ug/ml (six wells for each concentration; three wells contained antigen and the other three contained sodium-potassium phosphate buffer). Then, 50 ul/well of avidin-alkaline phosphatase (2 ug/ml) were added, followed by substrate after one hour. The P/N ratios were calculated as the ratio of the average absorbance in the presence of antigen to that in the absence of antigen.

To optimize the concentration of avidin-labeled alkaline phosphatase, antigen and biotin labelled antibody at optimum concentrations were added to an ELISA plate sequentially. Dilutions of avidin-alkaline phosphatase at 0.5, 1, 2, 3, 4,
5, and 6 ug/ml were added to each of 6 wells per concentration. Three wells contained antigen, the other three wells contained 0.05 M sodium-potassium phosphate buffer. The P/N ratio was measured as the ratio of the average absorbance in the presence of antigen to that in the absence of antigen.

Simultaneous and consecutive competition ELISA

Simultaneous and consecutive competition ELISA were used as described by Kubanek (1987). Immulon I ELISA plates were coated with antigen (50 ul/well) at the optimum concentration and blocked with 5% blotto. In the simultaneous assay, serial two-fold dilutions of unlabeled antibody (flask media) ranging from undiluted to 1:1024 were added (50 ul/well) together with biotinylated antibody (50 ul/well) at the optimum concentration. In some cases, the competitive ability of unlabeled antibodies was not exhausted at 1:1024 dilution. Therefore, another set of dilutions was used which included 1:5, 1:10, 1:50, 1:100, 1:250, 1:500, 1:1000, 1:5000, 1:25000 and 1:10000. In the consecutive assay, these two reagents were added stepwise; i.e., after incubation with unlabeled antibody at 37 C for one hour followed by washing, labeled antibody was added to the plates which were then incubated at 37 C. Then, 50 ul/well avidin-labeled alkaline phosphatase at the optimum concentration was added, followed by the addition of substrate solution. In these assays, plates were incubated at 37 C for 1 hour after addition of each reagent and washed
with wash buffer three times. The reaction products were measured at 410 nm.

In both assays four wells, which contained all the reagents except the unlabeled antibody, were used as positive controls. These were designated as having maximum binding (100% absorbance). Four other wells, containing all the reagents except antigen (for which 0.05 M phosphate buffer, pH 7.0 was substituted) and unlabeled antibody, were used as a negative control to assess whether the biotinylated antibody reacted non-specifically with the blocking agent.

The competition between labeled and unlabeled antibody for binding to antigen was assessed by calculating the percent maximum absorbance (PMA). Since the positive control was designated at 100% absorbance, the maximum absorbance would be reduced if the unlabeled antibody competed with the labeled antibody. The PMA was equal to the average absorbance of the sample divided by the average absorbance of the positive control.

It was assumed that the unlabeled antibody did not compete with the biotinylated antibody if, in both assays, PMA was greater than 75% at all dilutions of unlabeled antibody. Furthermore, greater competition in the simultaneous assay indicated that concentration of the unlabeled antibody had a greater effect on its ability to compete, while greater competition in the consecutive assay indicated that affinity had a greater impact. The results of these two assays also
provided evidence for the number of binding sites that the unlabeled antibody competed for on the antigen. When the consecutive and/or simultaneous assay curves were diphasic, the antibodies probably bound to two sites; if both the consecutive and simultaneous assay curves were monophasic, the antibodies competed for one site.

Antigens in competition ELISAs included CPSMV, serotypes I, II and III. MAbs used in competition assays included antibodies 6A, 5B, E2, F1, G10 and H6 produced against CPSMV I by Dr. J. H. Hill, Iowa State University, Ames, IA; 2-7A, 2-11A, 4-5B, 3-9C, 3-11C, 2-1F, 4-5F, 4-6F, 1-9G, 1-11G, 2-11G produced against CPSMV II by C. A. Kubanek; and 5B5, 6B3, 5C6, 4D8, 3E7, 2H10 produced against CPSMV III by the author.

In the first set of competition assays, CPSMV I was the coating antigen. MAb 5B was selected and biotinylated, because it had a very strong binding capacity to CPSMV I as determined in competition assays (Kubanek, 1987).

In the second set of competition assays, CPSMV II was the antigen and MAb 4-5B was used as the biotinylated antibody because it did not compete with MAb 5B in the first set of assays.

In the third set of competition assays, CPSMV III was the antigen. MAb 5B5 was the labeled antibody since it did not compete with MAbs 5B and 4-5B.

Based on the competitive characteristics of a total of 23 MAbs in simultaneous and consecutive competition assays, seven
MAbs (5B, 6A, 4-5B, 4-6F, 5B5, 4D8, 5C6) were chosen to form a panel for the signature analysis assays. However, MAbs 6A, 4-6F, 4D8 and 5C6 were not labeled with biotin and assayed in the simultaneous and consecutive competition ELISAs like MAbs 5B, 4-5B and 5B5. In order to further elucidate the competitive abilities of the seven MAbs used in the panel against each other, a simple antibody competition assay was performed. Fifty microliter per well of 15 ug/ml CPSMV I or II and 10 ug/ml CPSMV III, as optimized previously, were added separately into Dynatech Immulon I plates. Plates were incubated at 37 C for one hour and washed. After blocking with 5% blotto, 50 ul/well of biotinylated antibodies were added at optimized concentrations (5 ug/ml of MAbs 5B, 6A, 4-5B, 4-6F, 5B5, 5C6; 6 ug/ml of MAb 4D8) to the wells which contained homologous virus serotypes (i.e., MAbs 5B, 6A into wells with CPSMV I; MAbs 4-5B, 4-6F into wells with CPSMV II; MAbs 4D8, 5C6 into wells with CPSMV III). Simultaneously, 50 ul/well of these seven unlabeled purified MAbs with the same concentrations as the biotinylated antibodies, were added into the wells containing labeled MAb 5B, 6A, 4-5B, 4-6F, 5B5, 4D8 or 5C6. After incubation at 37 C for one hour and washing, 3 ug/ml avidin-labeled alkaline phosphatase was applied to the wells followed by the alkaline phosphatase substrate.
Signature Analysis

In the signature analysis assays by Wands et al. (1984), polystyrene beads were used as the solid phase, and the MAbs were directly labeled with $^{125}$I to generate signals. However, the $^{125}$I-labeling (Iodo-beads, Enzymobead and chloramine T methods) of MAbs to CPSMV I, II, III was not successful (author's unpublished results). The primary reason was that all the MAbs were IgM, and they lost immunoreactivity after labeling with $^{125}$I as determined by testing immunoreactivity of the $^{125}$I-labeled MAb by indirect ELISA. In addition, polystyrene beads did not seem to be an effective solid phase for the binding of antibodies and CPSMV antigens. Therefore, a modification of the signature analysis procedure was performed.

MAb 4D8 was selected as the capture antibody, because it was shown to give the highest reading (binding ability) among the seven antibodies for binding to CPSMV I, II, III and IV in an indirect ELISA test. The optimum concentration of MAb 4D8 was determined by diluting ascitic fluid of MAb 4D8 from 1:2 to 1:2$^{26}$ in 0.01 M PBS (pH 7.4). Dilutions were added to Dynatech Immulon I removawell strips (# 011-010-6301, 50 ul/well) which were incubated at room temperature overnight. After blocking with 5% blotto, 50 ul of of CPSMV I infected cowpea (diluted to 1:2$^{16}$) and healthy cowpea extracts were applied. Phosphate buffer (sodium-potassium phosphate buffer, 0.05 M, pH 7.0) was added as a control. After incubation at
37 C and washing, 5 ug/ml of biotinylated MAb 6A diluted in 1X wash buffer was added to the plate, followed by 200,000 cpm
$^{125}$I-labeled avidin (Amersham Corporation, Arlington Heights, IL; # IM.236). The strips were washed with wash buffer by using a Pentawash (Abbott Laboratories, North Chicago, IL.). Single wells were separated and transferred into glass tubes. The bound radioactivity (cpm) was counted with a gamma counter (Tracor Analytic Model 1191 Automatic Gamma System) and the P/N ratio was calculated.

The optimum concentrations for seven MAbs used in signature analysis were determined as in the simultaneous and consecutive competition ELISAs.

For the signature analysis assay, 50 ul of a 1:4000 dilution of MAb 4D8 ascitic fluid (in 0.01 M PBS, pH 7.0) was added to each well of the reproval strips. The strips were incubated at 22 C overnight, washed and blocked with 300 ul/well of 5% blotto. After incubating one hour at 37 C and washing, serial (two-fold) dilutions (1:2, 1:4, ..., 1:2048; i.e., 1:2^1, ..., 1:2^11) of fresh CPSMV-infected leaf extracts in sodium-potassium phosphate buffer were added at 50 ul/well. The extracts were prepared fresh each time by grinding one gram of infected (CPSMV I to IX) leaves in 1 ml of 0.05 M sodium-potassium phosphate buffer, and straining through two layers of cheese-cloth (the undiluted sample). Extracts from uninoculated cowpea leaves were prepared in the same way for use as a negative control. The strips were incubated at 37 C
for one hour and then washed with wash buffer. Seven biotinylated MAbs 5B, 6A, 4-5B, 4-6F, 5B5, 4D8 and 5C6 were added to the wells (50 ul/well) separately at 5 ug/ml in wash buffer. After incubation at 37 C for 1 hour, the strips were washed and 200,000 cpm/well of $^{125}$I-labeled avidin in wash buffer was added. After incubation at 22 C for one hour, wells were washed by using a Pentawash and the wells were separated and the bound radioactivity was counted with a gamma counter. In every experiment, the antigenic signatures of each antigen were generated simultaneously with seven MAbs in three replicates. These signatures were replicated three times with different samples, i.e., with three different preparations of the virus antigen.

For each serotype of CPSMV, binding curves (signatures) were established, with seven MAbs, by plotting LN (CPM/CK) (mean CPM bound in the test sample / mean CPM bound in healthy control by the dilution equivalent to the check) versus the exponential values of two-fold dilutions of antigen. Since the absolute concentration in each virus sample was not standardized, a statistical (iterative least-squares) technique was used to estimate the multiple response functions between LN (CPM/CK) and dilution factors. The statistical methods used in the iterative alignment and curve-fitting procedures have been described in detail by Ben-Porath et al. (1985). Data, LN (CPM/CK) and exponential values of two-fold dilution of antigens, were analyzed by a computer program, and
computer-graphic plots of aligned binding curves were generated. When aligned binding curves of two or more serotypes of CPSMV with a MAb were superimposable, they were considered to have a quantitatively and qualitatively identical epitope.
RESULTS

Purification of Cowpea Severe Mosaic Virus

The yields of CPSMV III obtained by differential and sucrose gradient centrifugation were very satisfactory. Yields ranged from 50.7 mg to 137 mg virus per kilogram of leaf tissue.

Production of Monoclonal Antibody

Two fusions were performed using CPSMV III as the immunogen. In the first fusion, about 70% of the wells contained fused cells, of which 20% produced antibodies specific to CPSMV III. After cloning with the limiting dilution procedure three times, four clones (2H10, 3E7, 5B5, 5C6) were selected which continued to secrete MAbs to CPSMV III. In the second fusion, hybridomas were cloned by using the calculation method and the two clones 4D8 and 6B3 were selected.

The selected clones were expanded in DMEM-R medium to produce flask media and were injected into mice to produce ascitic fluid. They were also frozen in DMSO medium and stored at -100 C.

Isotyping of Monoclonal Antibody

The class and subclass of MAbs produced by the selected cell lines were determined by the isotyping kit from Zymed
Laboratories. All 23 MAbs used in competition ELISAs produced IgM.

**Purification of Antibodies**

The yield of MAbs purified from 1 ml of ascitic fluid by precipitation with 0.32 M boric acid was approximately in the range from 0.5 mg to 5 mg IgM while the yield of antibodies purified on an A-mouse IgM affinity column ranged from 0.06-2 mg IgM/ml ascitic fluid.

**Competition Enzyme-Linked Immunosorbent Assays**

The optimum concentration of coating antigens, biotinylated antibodies and avidin-alkaline phosphatase were determined by calculating the P/N ratios in indirect ELISA. In the three sets of competition ELISAs, they were 15 ug/ml CPSMV I, 5 ug/ml biotinylated MAb 5B and 3 ug/ml avidin-alkaline phosphatase; 15 ug/ml CPSMV II, 5 ug/ml biotinylated MAb 4-5B and 5 ug/ml for avidin-alkaline phosphatase; and 10 ug/ml for CPSMV III (Fig. 1), 5 ug/ml for biotinylated MAb 5B5 and 2 ug/ml for avidin-alkaline phosphatase.

Results of competition experiments for seven MAbs used in the panel are summarized in Table 1. They were results of at least two independent experiments. The competition curves for these seven MAbs are shown in Figs. 2 to 8. Competition curves for the other MAbs are shown in Appendix A.

From the results of simultaneous and consecutive
Figure 1. Determination of the optimum virus concentration (CPSMV III) for competition ELISA assays. This was determined by calculating the P/N ratio (▲—▲) which was the ratio of the absorbance of the positive control (●—●) divided by negative control (●—●)
Table 1. Competition between monoclonal antibodies for binding sites on CPSMV I, II and III

<table>
<thead>
<tr>
<th>Unlabeled MAb</th>
<th>CPSMV I + 5B</th>
<th>CPSMV II + 4-5B</th>
<th>CPSMV III + 5B5</th>
<th>Competition / Shape of Curvea</th>
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<td>5B</td>
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<td>+ / 1</td>
<td></td>
</tr>
<tr>
<td>3E7</td>
<td>+ / 1</td>
<td>+ / 2</td>
<td>+ / 2</td>
<td></td>
</tr>
<tr>
<td>4D8</td>
<td>+ / 1</td>
<td>- / 2</td>
<td>+ / 1</td>
<td></td>
</tr>
<tr>
<td>5C6</td>
<td>+ / 2</td>
<td>+ / 2</td>
<td>+ / 2</td>
<td></td>
</tr>
<tr>
<td>6B3</td>
<td>+ / 1</td>
<td>+ / 2</td>
<td>+ / 1</td>
<td></td>
</tr>
</tbody>
</table>

aShape of competition curves is designated as (1) monophasic if both simultaneous and consecutive curves are monophasic; or as (2) if the simultaneous and/or consecutive curve is diphasic.

b+ indicates that the unlabeled MAb competed with biotinylated MAb for sites on the antigen in at least one competition (simultaneous or consecutive) assay.

c- indicates that the unlabeled MAb did not compete with biotinylated MAb in both simultaneous and consecutive competition assays.
Figure 2. Competition profiles of mAb 5B with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 5B(1): competition between 5B and labeled 5B using CPSMV I. 5B(2): competition between 5B and labeled 4-5B using CPSMV II. 5B(3): competition between 5B and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 3. Competition profiles of MAb 6A with biotin-labeled MAbs in simultaneous (•—•) and consecutive (←→) competition ELISAs. MAbs were made to CPSMV. 6A(1): competition between 6A and labeled 5B using CPSMV I. 6A(2): competition between 6A and labeled 4−5B using CPSMV II. 6A(3): competition between 6A and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 4. Competition profiles of MAb 4-5B with biotin-labeled MAbs in simultaneous (●—●) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 4-5B(1): competition between 4-5B and labeled 5B using CPSMV I. 4-5B(2): competition between 4-5B and labeled 4-5B using CPSMV II. 4-5B(3): competition between 4-5B and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 5. Competition profiles of MAb 4-6F with biotin-labeled MAbs in simultaneous (●—●) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 4-6F(1): competition between 4-6F and labeled 5B using CPSMV I. 4-6F(2): competition between 4-6F and labeled 4-5B using CPSMV II. 4-6F(3): competition between 4-6F and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 6. Competition profiles of MAb 5B5 with biotin-labeled MAbs in simultaneous (•--•) and consecutive (4--4) competition ELISAs. MAbs were made to CPSMV. 5B5(1): competition between 5B5 and labeled 5B using CPSMV I. 5B5(2): competition between 5B5 and labeled 4-5B using CPSMV II. 5B5(3): competition between 5B5 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 7. Competition profiles of MAb 4D8 with biotin-labeled MAbs in simultaneous (•—•) and consecutive (←→) competition ELISAs. MAbs were made to CPSMV. 4D8(1): competition between 4D8 and labeled 5B using CPSMV I. 4D8(2): competition between 4D8 and labeled 4-5B using CPSMV II. 4D8(3): competition between 4D8 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure 8. Competition profiles of MAb 5C6 with biotin-labeled MAbs in simultaneous (●—●) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 5C6(1): competition between 5C6 and labeled 5B using CPSMV I. 5C6(2): competition between 5C6 and labeled 4-5B using CPSMV II. 5C6(3): competition between 5C6 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Antibody Dilution ($2^{-x+1}$)

5C6 (1)

5C6 (2)

5C6 (3)
competition ELISAs, seven MAbs were chosen for use in signature analysis assays. They were MAbs 5B, 6A, 4-5B, 4-6F, 5B5, 4D8 and 5C6. MAbs 6A, 4-6F, 4D8 and 5C6 were not biotinylated and used in simultaneous and consecutive competition ELISAs. In order to further characterize the competitive ability of these seven MAbs, a simple competition assay was carried out. The results are summarized in Table 2.

**Signature Analysis**

The concentration of the capture MAb 4D8, determined by calculating the maximum P/N ratio for the response of the CPSMV I infected samples to that of the uninocualted cowpea samples (Figure 9), was optimal at a 1:4000 dilution of ascitic fluid.

The concentration for all seven biotinylated antibodies (5B, 6A, 4-5B, 4-6F, 5B5, 4D8, 5C6) used in signature analysis was chosen as 5 μg/ml because it was the optimum concentration for six out of seven antibodies, as determined by calculation of P/N ratios in simultaneous and consecutive competition ELISAs.

The antigenic signature for each serotype of CPSMV was generated with three different virus preparations (Appendix B). Antigenic signatures of all nine (I to IX) serotypes of CPSMV are shown in Figures 10-45. When each two of nine serotypes of CPSMV are directly compared, each serotype shows distinct binding profiles at multiple epitopes which are
Table 2. Competition between seven monoclonal antibodies as determined by antibody competition ELISAs

<table>
<thead>
<tr>
<th>Unlabeled MAb</th>
<th>CPSMV I</th>
<th>CPSMV II</th>
<th>CPSMV III</th>
</tr>
</thead>
<tbody>
<tr>
<td>5B</td>
<td>++(^a)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>6A</td>
<td>+</td>
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<td>+ ++</td>
</tr>
<tr>
<td>5C6</td>
<td>+</td>
<td>+</td>
<td>+ ++</td>
</tr>
</tbody>
</table>

\(^a\)Competition of unlabeled MAbs by 30-45\% (+), 45-60\% (++), 60-80\% (+++) against biotinylated MAbs for antigenic sites on CPSMV I, II and III.
Figure 9. Optimization of the concentration of capture MAb 4D8 ascitic fluid for signature analysis by ELISA. Five μg/ml of biotinylated MAb 6A and 200,000 cpm $^{125}$I-labeled avidin were used for the signal. P/N ratio was calculated as the mean cpm of CPSMV I sample divided by the mean cpm of the healthy cowpea control.
Figure 10. Antigenic signatures of CPSMV I (the solid line) and CPSMV II (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 11. Antigenic signatures of CPSMV I (the solid line) and CPSMV III (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV I+III/5B  
CPSMV I+III/6A  
CPSMV I+III/4-5B  
CPSMV I+III/4-6F  

Dilution of Antigen  

CPSMV I+III/5B5  
CPSMV I+III/4D8  
CPSMV I+III/5C6  

Dilution of Antigen
Figure 12. Antigenic signatures of CPSMV I (the solid line) and CPSMV IV (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 13. Antigenic signatures of CPSMV I (the solid line) and CPSMV V (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV 1+V/5B
CPSMV 1+V/6A
CPSMV 1+V/4-5B
CPSMV 1+V/4-6F

Dilution of Antigen

CPSMV 1+V/5B5
CPSMV 1+V/4D8
CPSMV 1+V/5C6

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen
Figure 14. Antigenic signatures of CPSMV I (the solid line) and CPSMV VI (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 15. Antigenic signatures of CPSMV I (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 16. Antigenic signatures of CPSMV I (the solid line) and CPSMV VIII (the dotted line) using MAb 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

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Dilution of Antigen
Figure 17. Antigenic signatures of CPSMV I (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV I+IX/5B

CPSMV I+IX/6A

CPSMV I+IX/4-5B

CPSMV I+IX/4-6F

CPSMV I+IX/5B5

CPSMV I+IX/4D8

CPSMV I+IX/5C6

Dilution of Antigen

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Dilution of Antigen
Figure 18. Antigenic signatures of CPSMV II (the solid line) and CPSMV III (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 19. Antigenic signatures of CPSMV II (the solid line) and CPSMV IV (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 20. Antigenic signatures of CPSMV II (the solid line) and CPSMV V (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 21. Antigenic signatures of CPSMV II (the solid line) and CPSMV VI (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV II+VI/5B
CPSMV II+VI/6A
CPSMV II+VI/4-5B
CPSMV II+VI/4-6F
CPSMV II+VI/5B5
CPSMV II+VI/4D8
CPSMV II+VI/5C6

Dilution of Antigen
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Dilution of Antigen

Dilution of Antigen
Dilution of Antigen
Dilution of Antigen
Figure 22. Antigenic signatures of CPSMV II (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 23. Antigenic signatures of CPSMV II (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV II+VIII/5B

CPSMV II+VIII/6A

CPSMV II+VIII/4-5B

CPSMV II+VIII/4-6F

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

CPSMV II+VIII/5B5

CPSMV II+VIII/4DB

CPSMV II+VIII/5GC

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Caution of Antigen Dilution of Antigen Dilution of Antigen Dilution of Antigen
Figure 24. Antigenic signatures of CPSMV II (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 25. Antigenic signatures of CPSMV III (the solid line) and CPSMV IV (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 26. Antigenic signatures of CPSMV III (the solid line) and CPSMV V (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 27. Antigenic signatures of CPSMV III (the solid line) and CPSMV VI (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 28. Antigenic signatures of CPSMV III (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Dilution of Antigen

CPSMV III+VII/58
CPSMV III+VII/6A
CPSMV III+VII/4-5B
CPSMV III+VII/4-6F

CPSMV III+VII/5B
CPSMV III+VII/4D8
CPSMV III+VII/5C6
Figure 29. Antigenic signatures of CPSMV III (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 30. Antigenic signatures of CPSMV III (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV III+IX/5B

CPSMV III+IX/6A

CPSMV III+IX/4-5B

CPSMV III+IX/4-6F

CPSMV III+IX/5B5

CPSMV III+IX/4D8

CPSMV III+IX/5C6

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen
Figure 31. Antigenic signatures of CPSMV IV (the solid line) and CPSMV V (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 32. Antigenic signatures of CPSMV IV (the solid line) and CPSMV VI (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV IV+VI/5B

CPSMV IV+VI/6A

CPSMV IV+VI/4-5B

CPSMV IV+VI/4-6F

CPSMV IV+VI/5B5

CPSMV IV+VI/4D8

CPSMV IV+VI/5C6

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen
Figure 33. Antigenic signatures of CPSMV IV (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 34. Antigenic signatures of CPSMV IV (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 35. Antigenic signatures of CPSMV IV (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
CPSMV IV+IX/5B
CPSMV IV+IX/6A
CPSMV IV+IX/4-5B
CPSMV IV+IX/4-6F

CPSMV IV+IX/5B5
CPSMV IV+IX/4D8
CPSMV IV+IX/5C6

Dilution of Antigen
Dilution of Antigen
Dilution of Antigen
Dilution of Antigen
Dilution of Antigen
Dilution of Antigen
Figure 36. Antigenic signatures of CPSMV V (the solid line) and CPSMV VI (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 37. Antigenic signatures of CPSMV V (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 38. Antigenic signatures of CPSMV V (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 39. Antigenic signatures of CPSMV V (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
<table>
<thead>
<tr>
<th>CPSMV V+IX/5B</th>
<th>CPSMV V+IX/6A</th>
<th>CPSMV V+IX/4-5B</th>
<th>CPSMV V+IX/4-6F</th>
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<td>Dilution of Antigen</td>
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</table>

Graphs showing dilution of antigen with varying dilutions and corresponding counts per minute (CPM).
Figure 40. Antigenic signatures of CPSMV VI (the solid line) and CPSMV VII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 41. Antigenic signatures of CPSMV VI (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 42. Antigenic signatures of CPSMV VI (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 43. Antigenic signatures of CPSMV VII (the solid line) and CPSMV VIII (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 44. Antigenic signatures of CPSMV VII (the solid line) and CPSMV IX (the dotted line) using MAbS 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
Figure 45. Antigenic signatures of CPSMV VIII (the solid line) and CPSMV IX (the dotted line) using MAbs 5B, 6A, 4-5B, 4-6A, 5B5, 4D8 and 5C6. Data are graphed with LN(CPM/CK) on the X-axis versus the exponential values of two-fold dilutions of antigen on the Y-axis.
summarized in Table 3. The difference between two serotypes on one epitope was measured by visualizing the divergence of two curves.
Table 3. Comparison of serotypes of CPSMV by signature analysis, showing epitopic differences recognized by seven MAbs

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<th>IV</th>
<th>V</th>
<th>VI</th>
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<th>VIII</th>
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a- or + indicates that there was no or some difference in the signature of the serotypes respectively.
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DISCUSSION

Cowpea (*Vigna unguiculata* Walp.) was an efficient host plant for the propagation of CPSMV. The yields of CPSMV were in the range of 50.7 mg-137 mg per kilogram of leaf tissue which agrees with the previous report of Lin et al. (1981).

MAbs were produced with a modified version of the Van Deusen and Whetstone's protocol (1981). Since hybrid cells, particularly in the early stages of proliferation, may lose chromosomes rapidly, some positive hybridomas may cease to produce antibody. Thus cloning of hybridomas is required (Milstein, 1980). In our hands, the cloned cells remained stable.

Limiting dilution and a calculation method were used for cloning. With the latter method, 30-40% of the wells had a single cell colony. With the limiting dilution approach, only 10-20% of the wells had single cell colony. The best way to clone hybridomas is by using a flow cytometer because of its accuracy and rapidity. It can separate single cells into individual wells of a 96-well tissue culture plate and therefore saves the tedious work of diluting and checking wells under the light microscope.

All the cell lines produced in this study were IgM-producers. This may be the result of the immunization schedule and the use of Freund's complete adjuvant. However, all the cell lines against CPSMV I and II produced by Hill and
Kubanek independently were also IgM-producers. Therefore, it is still unknown if the viral antigen plays any role in these results.

The purification of IgM has traditionally been more difficult than that of IgG. In this study, monoclonal IgM was purified from mouse ascitic fluid because ascitic fluid contains much more antibody than the culture supernatant (flask media) of hybridomas. As stated by Goding (1983), typical antibody concentrations in ascitic fluid range from 2 to 20 mg/ml while antibody concentrations in flask media range from 5 to 50 ug/ml. Our results are in good agreement with these observations.

Monoclonal IgM was biotinylated in order to perform competition ELISAs. Previous reports indicated that IgG could be successfully labeled with 1:50 v/v and 5:1 mol/mol ratio of biotin to IgG (Bayer et al., 1979). Since IgM is five times bigger than IgG, IgM was labeled with biotin in a 1:50 v/v and 20:1 mol/mol ratio of biotin to IgM in this study.

Our competition ELISAs were designed to reveal whether the affinity or the concentration of a MAb has the most significant impact on its competition with a labeled antibody, and to provide information regarding the putative number of antigenic sites on the antigen for which the MAb competes. In simultaneous competition assays, the ability of an unlabeled antibody to compete with a labeled antibody is primarily affected by its concentration. The unlabeled antibody is
prepared in a series of dilutions and the concentration of labeled antibody remains constant. The two immunoreagents are added to the wells simultaneously and compete for antigenic sites on the antigen at the same time. Thus, at high concentrations of unlabeled antibody, more unlabeled antibody will bind to the antigen and competition will be greater. As the concentration of unlabeled antibody decreases, more labeled antibody binds to the antigen and competition declines. Affinity of the unlabeled antibody also plays an important role in competition in the simultaneous assays. If the unlabeled antibody and biotinylated antibody have the same affinity, competition will be approximately 50% because both antibodies have an equal chance of binding to epitopes on the antigen.

Flask media were used as the source for the unlabeled MAbs. Although the IgM concentrations were not determined, they gave detectable absorbance readings in the antibody-containing indirect ELISA tests. As Goding stated (1983), antibody levels in flask media are usually in the range of 5-50 ug/ml. All the three biotinylated antibodies (5B, 4-5B, 5B5) had an optimum concentration of 5 ug/ml for use in the competition assays. Therefore, it is possible that the undiluted unlabeled antibody had at least the same concentration as the labeled antibody. If no competition occurred when the flask medium containing the unlabeled antibody was undiluted, it was not because there was
insufficient antibody; rather, it was due to the low affinity of the unlabeled antibody for the epitope.

In consecutive competition ELISA, however, the ability of unlabeled antibody to compete with a labeled antibody is primarily affected by affinity of the unlabeled antibody (Hosang, 1985). According to Berzofsky and Berkower (1984), the basic thermodynamic principles of antigen-antibody interactions are the same as those for any reversible bimolecular binding reaction. Its chemical equilibrium is formulated as $S + L \rightleftharpoons SL$, where $S = $ antibody binding sites, $L = $ ligand (antigen) binding sites, $SL = $ the complex of the two, and $K = $ association constant (or affinity). In the consecutive assay, the unlabeled antibody is added to the well before the biotinylated antibody. If the unlabeled antibody has a high affinity, the equilibrium equation favors binding of the unlabeled antibody to the antigen. As a result, the binding of unlabeled antibody to binding sites will be favored over binding of biotinylated antibody applied later. Therefore, in this situation, the unlabeled antibody competes with the labeled antibody even at high dilutions, to a fairly significant degree. Conversely, if the unlabeled antibody is of low affinity, the equilibrium equation favours dissociation of antigen-antibody complex. More biotinylated antibody will replace the unlabeled antibody at the binding sites. Therefore, competition of the unlabeled antibody is lower.

In analysis of competition curves for competitive
ability, both steepness and shape of the slopes are relevant as well as percent maximum absorbance values (Kubanek, 1987). In the simultaneous assay a steep slope suggests concentration of the unlabeled antibody does not have a large impact on its competition. If the slope increases gradually, the concentration influences competition. The consecutive assay is interpreted in a similar fashion. If the slope of the curve is steep, the data indicate that the antibody has a low affinity for the epitope and its affinity has little influence upon ability to compete. On the other hand, if the curve has a steep slope, the affinity of the unlabeled antibody has a significant impact upon its competition.

The shape of the competition curve suggests the putative number of binding sites that the unlabeled antibody competes for on the antigen. The curve is generated as a regression line of the third order. Thus, if it is a monophasic curve, the unlabeled antibody probably competes for one site on the antigen. If the curve is diphasic, it indicates that the unlabeled antibody may compete with the labeled antibody for binding to two different antigenic sites.

The percent maximum absorbance (PMA) values of both the simultaneous and consecutive assays (in a reasonable dilution range, from undiluted to 1:500) were also analyzed. If the PMA is lower in the consecutive than in the simultaneous assay, affinity is considered to have a greater impact than antibody concentration upon the ability of an antibody to
compete with the biotinylated antibody. If the PMA of the simultaneous assay is lower than the one in consecutive assay, the concentration is assumed to have more influence on the ability of the antibody to compete.

By these criteria, the 23 MAbs utilized in this study were grouped into two major categories of those that did compete and those that did not. In the competition ELISA which employed CPSMV I as the antigen, MAbs G10, 4-5B, 3-11C, 4-6F and 5B5 did not compete with biotinylated 5B because the PMA in both the simultaneous and consecutive assays were above 75%. The remainder of the MAbs were assumed to compete. In the second set of competition assays with biotinylated 4-5B and CPSMV II, MAbs E2, 4-6F, 1-11G, 5B5 and 4D8 did not compete. In the third group of competition assays using CPSMV III, all the antibodies competed with biotinylated 5B5. Competition of the unlabeled antibodies with the biotinylated antibody was assessed by competition in either the simultaneous or the consecutive assay.

Seven of the 23 MAbs were selected for use in signature analysis assays; therefore, only their characteristics are discussed below:

5B

MAb 5B was made against CPSMV I. It was selected for biotinylation because it had a very high capacity to bind to CPSMV I as determined by Kubanek (1987). The competition curves using CPSMV I as the antigen showed that MAb 5B
competes with biotinylated 5B. Evidently, concentration has a greater impact than affinity on competition, because the slope of the curve from the simultaneous assay was less steep than that from the consecutive assay. In addition, competition was lost at a dilution of 1:1000 in the simultaneous assay. No competition occurred above a dilution of 1:50 in the simultaneous assay. The antibody only bound to one site on CPSMV I.

The second set of competition curves demonstrated that the affinity of 5B was more significant in competition with biotinylated 4-5B than was concentration. It did not show competition, even when undiluted, in the simultaneous assay. It appeared to compete for two sites on CPSMV II, since it had a diphase consecutive competition curve [Figure 2, 5B(2)].

MAb 5B competed for binding to two sites on CPSMV III with labeled 5B5. Although the steepness of two curves did not suggest much difference, concentration was more significant in competition against labeled 5B5, because its PMA was from 30% to 75% in the dilution range of undiluted to 1:512.

Therefore, MAb 5B was chosen for the signature analysis because it had a relatively high binding capacity, it was the biotinylated antibody in competition assays and it could also bind to two sites on CPSMV III.

6A

MAb 6A was made to CPSMV I. It did not compete well with
biotinylated 5B for sites on CPSMV I because the PMA in both assays was around 75% at all dilutions of flask medium. It appeared to bind to two sites on CPSMV I.

The second set of competition curves suggested MAb 6A competed slightly with labeled MAb 4-5B. No competition was evident in the simultaneous assay and competition only occurred to a 1:16 dilution in the consecutive assay. It competed slightly for one site on CPSMV II.

MAb 6A competed with biotinylated 5B5 for sites on CPSMV III. Its affinity and concentration seem to play an equal role in its competitive ability because the steepness of both curves was similar. The PMA was below 75% at a dilution of flask medium as high as 1:128 in both assays. It competed with labeled 5B5 for binding to two sites on CPSMV III.

It was selected for signature analysis. Although the antibody was made to CPSMV I, it did not compete well with MAb 5B, i.e., it may bind to a different epitope on CPSMV I. This site may be similar to a site on CPSMV III, since it competed well against 5B5 on CPSMV III.

4-5B

MAb 4-5B, a MAb made to CPSMV II, was selected to be biotinylated in the second set of competition ELISAs because it did not compete with labeled 5B for sites on CPSMV I, and it showed a higher capacity for binding to CPSMV II in an indirect ELISA.

The second set of competition curves showed that affinity
and concentration of MAb 4-5B affected its competition more or less to the same degree. The slope of the curve in the simultaneous assay was slightly less steep than that of the consecutive curve. However, the competition was lost at a dilution of 1:128 in the consecutive assay and at 1:64 in the simultaneous assay. It bound to two sites on CPSMV II.

In the third set of competition assays, affinity and concentration of MAb 4-5B again seemed to have similar effects on its competitive ability. The slope of the curve was similar and it lost competition at about the same dilution (1:64-1:128). It also competed for two sites on CPSMV III.

MAb 4-5B was selected for signature analysis because it did not compete with MAb 5B for sites on CPSMV I. However, it competed very well with MAb 5B5 for sites on CPSMV III. Therefore, there may be a similar epitope on CPSMV II and III which was recognized by MAb 4-5B.

4-6F

MAb 4-6F, raised against CPSMV II, did not compete with both biotinylated 5B and 4-5B for antigenic sites on CPSMV I and II.

It competed well with labeled 5B5 for sites on CPSMV III. The slope of the binding curves for both simultaneous and consecutive assays was similar; the PMA of the simultaneous binding curve suggested that concentration had a greater impact than affinity on its competition. It competed with MAb 5B5 for binding to two sites on CPSMV III.
MAb 4-6F was chosen for signature analysis, because of its unique non-competitive nature with 5B and 4-5B. Since it was a MAb raised to CPSMV II, there may be an identical or similar epitope on both CPSMV II and III.

5B5

MAb 5B5 is a MAb made to CPSMV III. It was chosen for biotinylation for the third set of competition ELISAs and signature analysis, because it did not compete with both biotinylated MAbs 5B and 4-5B and it showed a very high binding ability to CPSMV III in an indirect ELISA. The competition curve against biotinylated MAb 5B5 showed that both concentration and affinity influenced its competition in both assays to a dilution of 1:512. It appeared to bind to one site on CPSMV III.

4D8

MAb 4D8 was made to CPSMV III. In the first competition assays, affinity influenced ability to compete with labeled 5B for sites on CPSMV I more than concentration. It did not compete beyond a 1:32 dilution of flask medium in the consecutive assay. The slope of the simultaneous curve was level with a constant PMA of about 70% at all dilutions. It competed with MAb 5B for one site on CPSMV I.

It did not compete with MAb 4-5B for sites on CPSMV II.

In competition with biotinylated 5B5 for sites on CPSMV III, the concentration of MAb 4D8 seemed to have a greater effect on ability to compete than affinity. The slope of the
two curves was very similar, but the PMA for the simultaneous assay was lower than that of consecutive assay. It bound to one site on CPSMV III.

It was selected for signature analysis, because it did not compete with MAb 4-5B on CPSMV II and it competed with MAb 5B on CPSMV I and with MAb 5B5 on CPSMV III. This suggested a similar epitope on CPSMV I and III.

MAb 5C6, an antibody raised to CPSMV III, did not compete well with MAb 5B for sites on CPSMV I because no competition occurred in the consecutive assay, and the PMA was 65% to over 75% in the simultaneous assay.

Competition with labeled MAb 4-5B for sites on CPSMV II did not occur beyond a 1:8 dilution of flask medium in the simultaneous assay, or beyond 1:32 in the consecutive assay. This indicated that affinity had a greater impact on competition with MAb 4-5B. It competed with MAb 4-5B for binding to two sites on CPSMV II.

MAb 5C6 competed well with biotinylated MAb 5B5 for sites on CPSMV III. With a relatively level consecutive competition curve and the ability to compete even at a dilution of 1:512, the affinity of MAb 5C6 definitely had a greater impact on its competition. It bound to two sites on CPSMV III.

It was chosen for signature analysis because unlike MAb 4D8, it did compete with MAb 5B, but not with MAB 4-5B. The two antibodies 5C6 and 4D8 may reveal some subtle differences
between epitopes of CPSMV I, II, III.

By employing a competition ELISA assay, Diaco et al. (1986) identified two epitopes on the coat protein of the P-PAV strain of BYDV. He demonstrated that two MAbs totally inhibited binding of the biotinylated antibody while one MAb had virtually no effect on binding of the biotinylated antibody. Competition ELISA assays with a small panel (2 to 3) of MAbs define only a limited number of epitopes on an antigen. They cannot demonstrate the subtle differences between a large number of epitopes. However, competition assays do provide a general idea concerning the relationships between epitopes. More importantly, they characterize MAbs by testing the impact of affinity and concentration on competition for epitopes on the antigen.

The MAbs characterized in this study were chosen for use in signature analysis assays to demonstrate the relationships of epitopes on strains of CPSMV. MAbs 5B, 6A, 4-5B, 4-6F, 5B5, 4D8 and 5C6 were selected on the basis of 1) ability to competitively bind to all three serotypes of CPSMV (5B, 6A, 5C6), and 2) to not compete with the biotinylated antibody and therefore recognize distinct and separate antigenic determinants on CPSMV (4-5B, 4-6F, 5B5, 4D8).

MAbs 6A, 4-6F, 4D8 and 5C6 were also biotinylated and used in a simple antibody competition assay together with biotinylated MAbs 5B, 4-5B and 5B5 to further characterize their competition (Table 2). The seven MAbs were shown to
compete with each other by 30 to 80% which agreed with results achieved in simultaneous and consecutive competition ELISAs in which four of the antibodies (i.e., 6A, 4-6F, 4D8 and 5C6) were not biotinylated.

Serotypes I through IX of CPSMV were assayed by signature analysis, although there were no antibodies directly produced against serotypes IV to IX. The data showed that these heterologous antibodies could properly define epitopes on CPSMV IV-IX. Our results reflected the interaction between antigenic epitopes and antibodies. When serotypes of CPSMV were compared, differences in the relative occurrence of each epitope as well as variations in affinity resulting from structural differences within the epitopes were reflected in the signatures. The signatures of each antibody with each antigen were generated by plotting the exponential values of two-fold dilution of antigen on the X-axis and LN(CPM/CK) on the Y-axis. The interaction of antigen at high concentrations with antibodies of relatively high affinity resulted in a relatively high signature curve. Since the affinity of an antibody to the epitope of an antigen was constant, the signature curve would gradually drop as the concentration of the antigen decreased. When the signature curves of two serotypes against one MAb were superimposable, showing no divergence, these two serotypes were considered to have one quantitatively and qualitatively identical epitope.

In our experiments, the antigen was prepared from freshly
infected leaves prior each experiment; therefore, the starting concentration of the antigens were probably different from one experiment to the other. The signature curves generated at different times would shift to the left or right if the starting antigen concentration was lower or higher, but the basic relationship of each curve to the other remained constant. Thus, a reproducible signature results (Van Deusen, 1984). The fresh antigen dilutions used in this study varied from 1:2 to 1:2048 (two-fold dilutions). Extracts from uninoculated cowpeas at the same dilutions were used as a control. Results (author's unpublished results) suggested that, at higher concentrations (1:2 to 1:8), the cowpea plant sap may interfere with interactions between virus antigens and biotinylated antibodies. However, the CPMs resulting from the interaction between biotinylated antibodies and healthy cowpea sap from 1:16 to 1:2048 dilution were approximately the same as the negative control (with phosphate buffer). Therefore, the interaction results between CPSMV serotypes and seven MAbs (sample CPM) were divided by uninoculated cowpea CPM at each equivalent dilution point, which were plotted on the Y-axis.

Figures 10 to 45 showed the results of signature analysis assays with CPSMV I through IX and seven MAbs as well as a comparison of two serotypes against each antibody. Each curve was the result of the average of three independent analyses with three replicates each time. In signature analysis, the absolute antigen concentration of each virus sample was not
standardized. Therefore, a statistical (iterative least-squares) technique (Ben-Porath et al., 1985) was used to align the curves generated with three CPSMV serotype (antigen) preparations. This allowed comparison of binding profiles and detection of both subtle and major antigenic differences. In this study, the antigenic signature differences were determined by visual assessment and scored "+" (major difference) and "-" (minute or no difference) (Table 3). However, statistical quantitation methods could be pursued to further demonstrate the differences of antigenic signatures among these nine serotypes of CPSMV.

When all serotypes of CPSMV were compared, each had distinct binding profiles at multiple epitopes. Table 3 illustrated the differences between serotypes of epitopes recognized by each of the seven MAbs. By immunodiffusion tests, Lin et al. (1981) showed that serotypes I to IV had a common antigenic determinant A. Serotypes I, III and IV shared an additional common determinant designated as B. Serotypes I and IV had a common epitope C. Each serotype also had a specific antigenic determinant D, E, F and G for serotypes I, II, III and IV, respectively. From Table 3, three common epitopes recognized by MAbs 5B, 6A and 4-5B were shared by CPSMV I, III and IV. But assumptions cannot be made to suggest that either epitope "5B", "6A" or "4-5B" was the determinant B identified with double diffusion tests. This is because the differences between serotypes recognized by
polyclonal antibodies reflect the differences of more than one epitope. In order to define the relationships between epitopes recognized by polyclonal antibodies and those by MAbs, double diffusion tests using side-by-side comparisons with both types of antibodies, could be utilized.

By vertical and horizontal comparisons of data in Table 3, the differences in reactivity of seven MAbs with nine CPSMV serotypes are easily identified. For example, Table 3 showed CPSMV II was very different from the other serotypes of CPSMV, which coincided the results obtained by Lin et al. (1984). In addition, Table 3 showed that CPSMV VIII was slightly different from CPSMV IX on only epitopes "5B" and "4-5B".

Since each labeled MAb and $^{125}$I-labeled avidin were used at identical protein concentrations, the affinity differences between virus serotypes could be measured. In Figure 10, the frequency of epitope 6A was higher than epitope 4-5B for CPSMV I. In Figure 10, the relative affinity of antibody 5B for its epitope on CPSMV III was higher than that on CPSMV I.

In this study, all antibodies were IgM. Biotin and avidin system instead of direct $^{125}$I-labeling had to be used in order to preserve the reactivity of the antibodies. This could be the reason that the differences among nine serotypes of CPSMV are not very striking. However, signature analysis has been proven to be able to differentiate nine serotypes of CPSMV. However, only seven MAbs were used in this study. In order to further elucidate the differences of CPSMV serotypes,
more MAbs should be included.

Signature analysis, as a relatively new technique, has not, until this study, been applied to plant virology. Its ability to define the subtle antigenic differences among animal viruses (Wands et al., 1984; Monath et al., 1986) and plant viruses (this study) has been well demonstrated. This technique will be most valuable in establishing signatures common to several isolates so that new strains (serotypes) may be compared to the prevalent strain (serotype) (Monath, 1986). This may allow comparison between strains (serotypes) derived from different geographic regions and permit classification of variant strains. It may be possible to trace the genetic evolution and antigen drift of plant viruses. This is important to plant virologists since we have not been able to label viruses as do the plant mycologists and bacteriologists. In addition, antigenic drift would possibly indicate that new serotypes of virus may have developed and they might be able to overcome resistant hosts. This could have implications for the breeding of resistant plants which is by far the most feasible way to control plant viral disease.


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Figure A1. Competition profiles of MAb E2 with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. E2(1): competition between E2 and labeled 5B using CPSMV I. E2(2): competition between E2 and labeled 4-5B using CPSMV II. E2(3): competition between E2 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A2. Competition profiles of MAb F1 with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. F1(1): competition between F1 and labeled 5B using CPSMV I. F1(2): competition between F1 and labeled 4-5B using CPSMV II. F1(3): competition between F1 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A3. Competition profiles of MAb G10 with biotin-labeled MAbs in simultaneous (●—●) and consecutive (4—4) competition ELISAs. MAbs were made to CPSMV. G10(1): competition between G10 and labeled 5B using CPSMV I. G10(2): competition between G10 and labeled 4-5B using CPSMV II. G10(3): competition between G10 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A4. Competition profiles of MAb H6 with biotin-labeled MAbs in simultaneous (•---•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. H6(1): competition between H6 and labeled 5B using CPSMV I. H6(2): competition between H6 and labeled 4-5B using CPSMV II. H6(3): competition between H6 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Antibody Dilution ($x^{-1}$)

% Maximum Absorbance

H6 (1)

H6 (2)

H6 (3)
Figure A5. Competition profiles of MAb 2-7A with biotin-labeled MAbs in simultaneous (•—•) and consecutive (•—•) competition ELISAs. MAbs were made to CPSMV. 2-7A(1): competition between 2-7A and labeled 5B using CPSMV I. 2-7A(2): competition between 2-7A and labeled 4-5B using CPSMV II. 2-7A(3): competition between 2-7A and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A6. Competition profiles of MAb 2-11A with biotin-labeled MAbs in simultaneous (•••) and consecutive (▲▲▲) competition ELISAs. MAbs were made to CPSMV. 2-11A(1): competition between 2-11A and labeled 5B using CPSMV I. 2-11A(2): competition between 2-11A and labeled 4-5B using CPSMV II. 2-11A(3): competition between 2-11A and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A7. Competition profiles of MAb 3-9C with biotin-labeled MAbs in simultaneous (•--•) and consecutive (•--•) competition ELISAs. MAbs were made to CPSMV. 3-9C(1): competition between 3-9C and labeled 5B using CPSMV I. 3-9C(2): competition between 3-9C and labeled 4-5B using CPSMV II. 3-9C(3): competition between 3-9C and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A8. Competition profiles of MAb 3-11C with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 3-11C(1): competition between 3-11C and labeled 5B using CPSMV I. 3-11C(2): competition between 3-11C and labeled 4-5B using CPSMV II. 3-11C(3): competition between 3-11C and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A9. Competition profiles of MAb 2-1F with biotin-labeled MAbs in simultaneous (•---•) and consecutive (•—•) competition ELISAs. MAbs were made to CPSMV. 2-1F(1): competition between 2-1F and labeled 5B using CPSMV I. 2-1F(2): competition between 2-1F and labeled 4-5B using CPSMV II. 2-1F(3): competition between 2-1F and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A10. Competition profiles of MAb 4-5F with biotin-labeled MAbs in simultaneous (•---•) and consecutive (•—•) competition ELISAs. MAbs were made to CPSMV. 4-5F(1): competition between 4-5F and labeled 5B using CPSMV I. 4-5F(2): competition between 4-5F and labeled 4-5B using CPSMV II. 4-5F(3): competition between 4-5F and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A11. Competition profiles of MAb 1-9G with biotin-labeled MAbs in simultaneous (●—●) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 1-9G(1): competition between 1-9G and labeled 5B using CPSMV I. 1-9G(2): competition between 1-9G and labeled 4-5B using CPSMV II. 1-9G(3): competition between 1-9G and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A12. Competition profiles of MAb 1-11G with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 1-11G(1): competition between 1-11G and labeled 5B using CPSMV I. 1-11G(2): competition between 1-11G and labeled 4-5B using CPSMV II. 1-11G(3): competition between 1-11G and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A13. Competition profiles of MAb 2-11G with biotin-labeled MAbs in simultaneous (●—●) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 2-11G(1): competition between 2-11G and labeled 5B using CPSMV I. 2-11G(2): competition between 2-11G and labeled 4-5B using CPSMV II. 2-11G(3): competition between 2-11G and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A14. Competition profiles of MAb 2H10 with biotin-labeled MAbs in simultaneous (•—•) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 2H10(1): competition between 2H10 and labeled 5B using CPSMV I. 2H10(2): competition between 2H10 and labeled 4-5B using CPSMV II. 2H10(3): competition between 2H10 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A15. Competition profiles of MAb 3E7 with biotin-labeled MAbs in simultaneous (•-•) and consecutive (▲-▲) competition ELISAs. MAbs were made to CPSMV. 3E7(1): competition between 3E7 and labeled 5B using CPSMV I. 3E7(2): competition between 3E7 and labeled 4-5B using CPSMV II. 3E7(3): competition between 3E7 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
Figure A16. Competition profiles of MAb 6B3 with biotin-labeled MAbs in simultaneous (■—■) and consecutive (▲—▲) competition ELISAs. MAbs were made to CPSMV. 6B3(1): competition between 6B3 and labeled 5B using CPSMV I. 6B3(2): competition between 6B3 and labeled 4-5B using CPSMV II. 6B3(3): competition between 6B3 and labeled 5B5 using CPSMV III. Percent maximum absorbance is equal to the average absorbance of the sample divided by the average absorbance of the positive control.
APPENDIX B
Figure B1. Antigenic signatures of CPSMV I determined by seven MAbs with three independent virus preparations. Three sets of data (---, --, --) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Figure B2. Antigenic signatures of CPSMV II determined by seven MAbs with three independent virus preparations. Three sets of data (--, --, --) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Figure B3. Antigenic signatures of CPSMV III determined by seven MAbs with three independent virus preparations. Three sets of data (---, ---, ---) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis
Figure B4. Antigenic signatures of CPSMV IV determined by seven MAbs with three independent virus preparations. Three sets of data (---, ---, ---) are graphed with $\ln(\text{CPM}/\text{CK})$ on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Dilution of Antigen

CPSMV IV / 5B

CPSMV IV / 6A

CPSMV IV / 4-5B

CPSMV IV / 4-6F

CPSMV IV / 5B5

CPSMV IV / 4D8

CPSMV IV / 5C6

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen

Dilution of Antigen
Figure B5. Antigenic signatures of CPSMV V determined by seven MAbs with three independent virus preparations. Three sets of data ( -- , -- , -- ) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Dilution of antigen
Figure B6. Antigenic signatures of CPSMV VI determined by seven MAbs with three independent virus preparations. Three sets of data ( -- , -- , -- ) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Figure B7. Antigenic signatures of CPSMV VII determined by seven MAbs with three independent virus preparations. Three sets of data (---, ---, ---) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.
Figure B8. Antigenic signatures of CPSMV VIII determined by seven MAbs with three independent virus preparations. Three sets of data ( -- , -- , -- ) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis
Dilution of antigen
Figure B9. Antigenic signatures of CPSMV IX determined by seven MAbs with three independent virus preparations. Three sets of data (---, ---, ---) are graphed with LN(CPM/CK) on the Y-axis versus the exponential values of two-fold dilutions of antigen on the X-axis.