ANALYSIS OF THE STRUCTURE AND BIOLOGICAL ACTIVITY OF *PRUNUS* NECROTIC RINGSPOT AND APPLE MOSAIC VIRUSES USING MONOCLONAL ANTIBODIES

by

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ABSTRACT

Monoclonal antibodies reactive with two ilarviruses, Prunus necrotic ringspot virus (PNRSV) and apple mosaic virus (ApMV) were utilized to determine the structural and biological properties of the viruses. Three monoclonal antibodies (Mabs 1, 2 and 3) which reacted only to ApMV in various ELISA tests were found to bind to conformation independent epitopes which are hidden between or within viral subunits. Three other monoclonal antibodies (Mabs 5, 6 and 7) which reacted only to PNRSV in various ELISA tests were found to bind to conformation dependent, externally located epitopes. One monoclonal antibody, Mab 4, which reacted with both viruses was found to bind to a conformation dependent, partially hidden epitope on ApMV, but a conformation independent, external epitope on PNRSV. Further, the three ApMV-specific antibodies reacted with viral coat protein in Western blots. None of the three PNRSV-specific antibodies reacted with viral coat protein in Western blots. Mab 4, however, did react with PNRSV coat protein but not ApMV coat protein in Western blots. Fragments of PNRSV coat protein were generated by proteolysis, and, in Western blots, all were found to be bound by Mab 4. Thus, attempts to isolate a specific polypeptide containing the epitope were unsuccessful. In neutralization of infectivity studies, Mab 4 blocked PNRSV infectivity although the antibody did not precipitate the virus in Ouchterlony double diffusion tests. This suggests that Mab 4 may bind to an epitope located in a region of the coat protein necessary for infection. Although not yet examined for PNRSV, a characteristic of other ilarviruses is coat protein dependent initiation of infection, where coat protein, or a subgenomic RNA which codes for coat protein, is required for the RNAs to be infectious. Thus, Mab 4 may be a useful probe for studying this process in PNRSV and other ilarviruses.

DEDICATION

I dedicate this thesis to my husband, Mark,
for his understanding and support.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ApMV	Apple mosaic virus
AMV	Alfalfa mosaic virus
CLRV	Citrus leaf rugose virus
DAS-ELISA	Double antibody sandwich ELISA
ELISA	Enzyme-linked immunosorbent assay
ID	Immunodiffusion test
Mabs	Monoclonal antibodies
PAGE	Polyacrylamide gel electrophoresis
PNRSV	*Prunus* necrotic ringspot virus
RMV	Rose mosaic virus
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline, pH 7.5
TSV	Tobacco streak virus
2-ME	2-mercaptoethanol

INTRODUCTION

Monoclonal antibodies have been utilized in numerous studies in all fields of molecular biology to determine the structure or activity of a molecule based on specific binding. They have recently been utilized in plant virus research, particularly with ELISA and Western blot techniques to analyze virus structure. The ilarviruses have been the subject of investigations using monoclonal antibodies produced by Halk et al. (1984). In the present study, the reactivity of these antibodies with two ilarviruses under varying experimental conditions was analyzed to determine virus structure and biological activity.

The ilarvirus group consists of about twenty mechanically transmissible viruses having quasi-isometric particles from 23 to 25 nm in diameter (Francki, 1985). They possess tripartite genomes, that is, by definition, three genomic single-stranded RNA molecules. There are, however, four RNA molecules termed RNAs 1, 2, 3 and 4. The RNA 4 has the same sequences as a portion of RNA 3, so is considered subgenomic. Each virus has one species of polypeptide of molecular weight 25,000-28,000 daltons. In sucrose density gradients, the virus sediments as three or four components. Particles are stabilized by RNA-protein interaction as opposed to protein-protein interaction.

Two members of the ilarviruses discussed in this report are Prunus necrotic ringspot virus (PNRSV) and apple mosaic virus (ApMV). Others mentioned are tobacco streak virus (TSV), citrus leaf rugose virus (CLRV) and rose mosaic virus (RMV). Rose mosaic virus is very closely related or even identical to ApMV. One other

virus related to the ilarviruses in physical properties is alfalfa mosaic virus (AMV) which is the sole member of its own group based on host range and aphid vectors. It is discussed here because of the extensive work that has been done on its RNAs and coat protein and its similarities to the ilarviruses.

Existing knowledge of Prunus necrotic ringspot and apple mosaic viruses

Prunus necrotic ringspot virus particles are quasi-isometric but approximately 22-23 nm in diameter (Francki, 1985; Fulton, 1970). In sucrose gradients they sediment as three components. The bottom (B) component contains RNA 1, the middle (M) component contains RNA 2, and the top (T) component contains either RNA 3 or two molecules of RNA 4 (Loesch and Fulton, 1975; Gonsalves and Fulton, 1977). Apple mosaic virus particles are also heterogeneous but slightly larger than PNRSV (Fulton, 1972). The early work on PNRSV as a multicomponent system (Loesch and Fulton, 1975) reports that M and T particles are not infectious, B particles are slightly infectious, M+B is infectious, but M+B+T results in a decrease in infectivity. Why the addition of T particles has that effect is unclear. However, the significance of this work is that the B component, consisting of the largest RNA surrounded by a protein coat, is required for infection. Gonsalves and Fulton (1977) determined that PNRSV RNAs and RMV (ApMV) RNAs each separate into four components in sucrose gradients. Termed RNAs 1, 2, 3 and 4 in order of decreasing sedimentation velocity, they were collected in two fractions, RNA 1+2+3 and RNA 4, recycled and used in infectivity assays. Each fraction alone was not infectious, but when combined,

infectivity was restored. In addition, the heterologous mixture of RMV RNA 1+2+3 and PNRSV RNA 4 was infectious, although PNRSV RNA 1+2+3 plus RMV RNA 4 was not.

The finding that RNA 4 activates RNA 1+2+3 is consistent with studies of two other ilarviruses, TSV (van Vloten-Doting, 1975) and CLRV (Gonsalves and Garnsey, 1975a) and for the related AMV (Bol et al., 1971). Also, RMV, TSV, CLRV and AMV were shown to exhibit the additional property of "functional equivalence" of coat protein and the small RNA, in which coat protein could be substituted for the small RNA in activating the mixture of larger RNAs. Furthermore, coat proteins are interchangeable among certain viruses as activators of infectivity (Gonsalves and Garnsey, 1975b). The property of coat protein dependent initiation of infection seems to be characteristic of the few ilarviruses tested to date, including RMV (ApMV) but has not yet been examined for PNRSV. The functional equivalence of coat protein and the small RNA could be explained since, in AMV, the subgenomic RNA contains the genetic information for coat protein (Jaspars, 1985). However, the function of the coat protein in initiating infection is unknown.

Rationale for using monoclonal antibodies to examine PNRSV and ApMV

Biochemical and immunochemical studies on the ilarviruses have been hampered by difficulties in working with these extremely labile viruses. The particles are stabile under precise buffering conditions in sap for only a few hours and in purified form for only one or two weeks (based on infectivity assays) (Fulton, 1970 and personal observation). Particle instability, in addition to variability inherent in polyclonal antisera production, results in

difficulties in obtaining good quality antisera. Polyclonal antisera also may contain antibodies to whole and fragmented virions. The latest approach to obtaining specific antibodies has been the production of hybridomas that secrete monoclonal antibodies (Kohler and Milstein, 1975). Monoclonal antibodies are valuable tools for determining virus structure based on whether or not an antibody binds to its epitope under varying conditions. For example, Tremaine et al. (1985) was able to show differential monoclonal antibody binding to swollen versus unswollen Southern bean mosaic virus particles indicating the location of the epitopes on the subunits. Monoclonal antibodies can also be utilized to identify specific regions of a protein, such as those generated by proteolytic digestion or those epitopes which are conformation dependent (Friguet et al., 1984; Schmaljohn et al., 1983).

Background on the ilarvirus monoclonal antibodies

Monoclonal antibodies (Mabs) against three ilarviruses and AMV were produced by Halk et al. (1982) and analyzed as serotyping reagents (Halk et al., 1984). These hybridomas were produced from a mouse immunized with a mixture of PNRSV, ApMV, TSV and AMV. Seven Mabs were produced which reacted in indirect ELISA tests with PNRSV and/or ApMV (also Jordan and Aebig, 1985, and Appendix B). As diluted ascitic fluid, Mabs 1, 2 and 3 reacted only with ApMV, Mabs 4 and 5 reacted with both viruses, and Mabs 6 and 7 reacted only with PNRSV. After purification, Mab 5 no longer bound to ApMV. All the Mabs (except 1 and 2) were shown to bind to different epitopes based on serotyping eight strains of ilarviruses in indirect ELISA. None of the Mabs reacting with ApMV precipitated ApMV in

immunodiffusion tests. Mabs 5, 6 and 7 all precipitated PNRSV. The exceptional antibody was Mab 4 which did not precipitate either virus. A summary of the reactivities of the seven Mabs with the viruses is presented in Table I.

The immunoreactvities of the Mabs with virus treated with SDS or SDS and 2-mercaptoethanol (2-ME) and spotted to nitrocellulose were tested to determine which epitopes are conformation independent (Appendix B; Appendix D, Fig. 1; Jordan, Aebig and Hsu, manuscript in preparation). Even at very low concentrations, SDS dissociates ilarvirus particles into subunits by interfering with protein-RNA linkages that stablize the virions (Kaper, 1973; Francki et al., 1985). 2-mercaptoethanol reduces and linearizes protein by breaking disulfide bonds, and, together with SDS, destroys protein conformation. After spotting to nitrocellulose and removing SDS and 2-ME, the protein may possess some conformational structure resulting from the amino acid sequence, but not secondary or tertiary structures. Generally, the ApMV-specific Mabs 1, 2 and 3 had stronger reactivities with dissociated virus than with untreated virus. These Mabs reacted with SDS, 2-ME-treated ApMV indicating the antibody-epitope bond is not dependent on viral subunits being in the native conformational state. Thus, the epitopes are conformation independent. Conversely, Mab 4 reactions were strongest with untreated ApMV and negative with dissociated virus, so the epitope is conformation dependent. The PNRSV-specific Mabs 5, 6 and 7 reacted stronger with untreated than dissociated virus (conformation dependent epitopes). Mab 4 reacted strongly with both untreated and dissociated PNRSV, and, since it reacted with SDS,

Table I. Summary of reactivities of monoclonal antibodies with apple mosaic virus (ApMV) and Prunus necrotic ringspot virus (PNRSV).[a]

	ApMV		PNRSV	
Monoclonal antibody	ELISA[b]	ID[c]	ELISA	ID
1	+	0	0	0
2	+	0	0	0
3	+	0	0	0
4	+	0	+	0
5	0	0	+	+
6	0	0	+	+
7	0	0	+	+

[a]Halk et al., 1984; Jordan, Aebig and Hsu, manuscript in preparation.

[b]Indirect ELISA. Concentration of antibody tested was 50 ug/ml. +, reactive. 0, non-reactive.

[c]Immunodiffusion test. Concentration of antigen tested was 50 ug/ml. +, reactive. 0, non-reactive.

2-ME-treated PNRSV, the epitope is conformation independent. Thus, Mabs 1-4 were selected as probes in Western blot analysis in the present study.

Rationale for using limited proteolysis for generating fragments

The technique of peptide mapping by limited proteolysis and analysis by polyacrylamide gel electrophoresis (PAGE) has been shown to be a rapid and convenient way of characterizing peptide fragments (Cleveland et al., 1977). The basis for this method is the fact that SDS-PAGE is a reliable method for determining molecular weights (Weber and Osborn, 1969) and that peptide fragments could be generated in a reproducible manner despite some variation in incubation time and substrate concentration. The technique has been used for virus isolate identification, structural analysis and epitope mapping. Edwards and Gonsalves (1983) used this technique to confirm that certain isolates of cucumber mosaic virus were in fact structurally different. Hiebert et al. (1984) used limited proteolysis to show heterogeneity among capsid proteins of five different potyviruses. Lastly, there have been numerous reports of using limited proteolysis in conjunction with ELISA or Western blotting to locate and identify epitopes (Heinz et al., 1983; Mackenzie and Tremaine, 1986).

Thesis Objectives

The objectives of this research are the following:

1. To determine the location of the epitopes on the virion. This will be accomplished using indirect and double antibody sandwich ELISA determination of monclonal antibody reactivity with untreated and SDS-dissociated viruses.

2. To show that some of the monoclonal antibodies react with PNRSV coat protein or ApMV coat protein as determined by Western blot analysis.

3. To fragment viral coat protein by proteolysis and using Western blot techniques identify which piece contains the Mab 4 epitope.

4. To determine if the monoclonal antibodies affect the biological properties of the virus by interfering with infectivity. This will be accomplished by performing neutralization of infectivity assays.

Preliminary reports have been presented [Jordan et al., 1985 (Appendix B) and Aebig et al., 1986 (Appendix C)], a manuscript has been submitted (Appendix D), and a manuscript is in preparation (Jordan, Aebig and Hsu).

MATERIALS AND METHODS

Virus Purification

In the studies of PNRSV and ApMV, extreme care was taken during purification procedures to obtain highly purified intact particles. Briefly, infected Lemon cucumbers were harvested four to five days after inoculation and the virus extracted by the hydrated calcium phosphate method, followed by polyethylene glycol precipitation, differential centrifugation and sucrose density centrifugation (Halk et al., 1984). Purified virus was aliquoted and the vials quickly frozen in liquid nitrogen and stored either in liquid nitrogen or at -70 C. In most cases, experiments were performed with virus thawed that day or occasionally with virus stored at 4 C for no more than one week. This insured that the virus preparation consisted of intact particles at the beginning of each experiment.

Monoclonal antibodies

Monoclonal antibodies were produced by Halk et al. (1984). The source of antibodies for these studies was ascitic fluid from which immunoglobulins were purified by ammonium sulfate precipitation, and, with some antibodies, column chromatography (Jordan and Aebig, 1985).

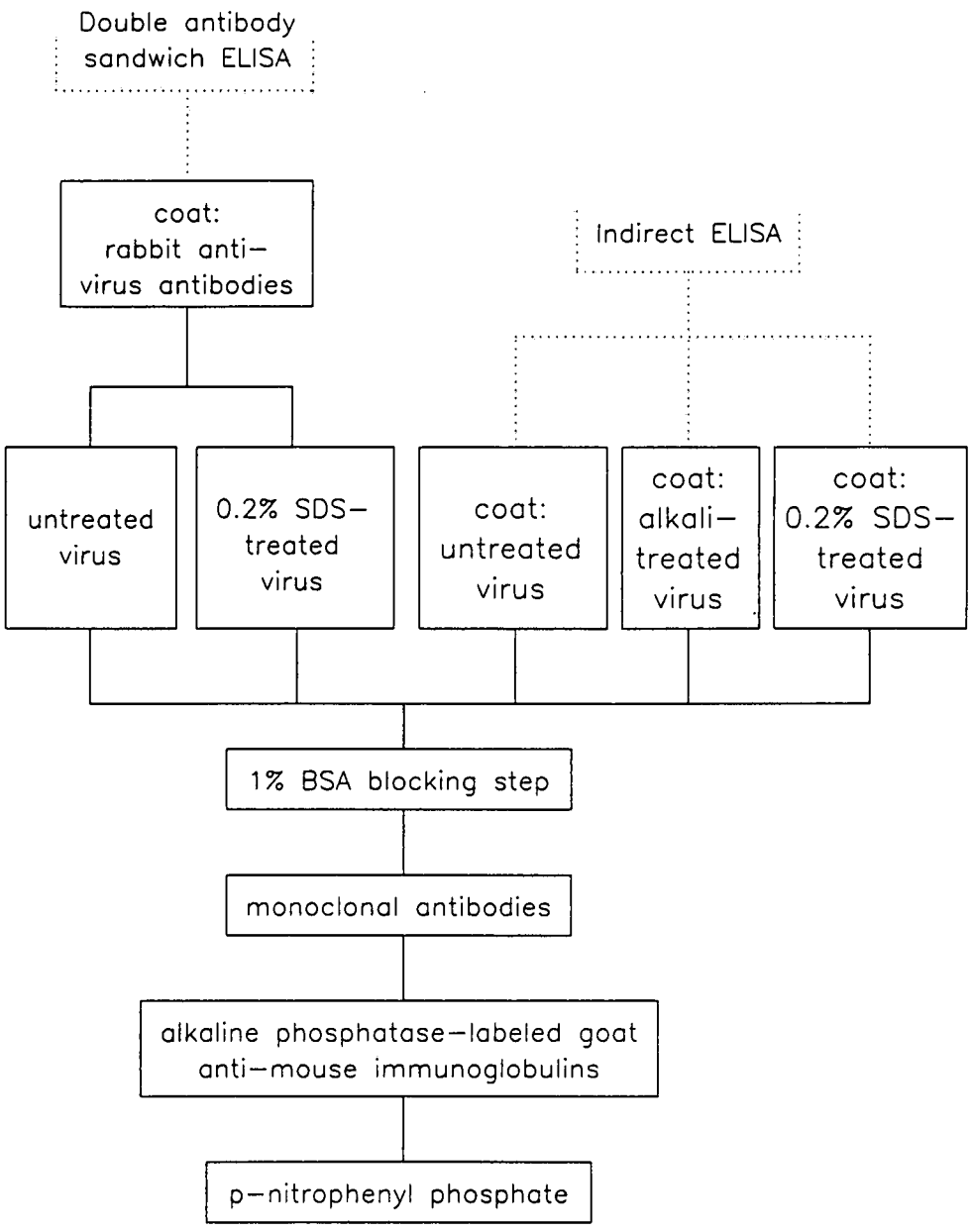
Polyclonal antibodies

Rabbit anti-virus antibodies were purified by ammonium sulfate precipitation from polyclonal antisera (ATCC PVAS 22 and PVAS 32).

ELISA

Five different ELISA tests were used to generate binding curves of monoclonal antibody reaction with each of the purified viruses (Fig. 1). In double antibody sandwich ELISA (DAS ELISA) virus was

Fig. 1 Process flow diagram of the five ELISA tests.



trapped by rabbit anti-virus antibodies coated onto polyvinyl chloride plates (50 ul/well) at 2 ug per ml in .02 M Tris, 0.15 M NaCl, pH 7.5 (TBS). The plates were incubated for 3.5 hr at room temperature and washed three times with TBS containing 0.05% Tween-20. Two different virus solutions were used for the next step. In one, newly thawed, untreated virus, at 2 ug/ml in TBS, was incubated on the rabbit antibody-coated plates. In the other, virus was first dissocated in a solution of 0.2% SDS in TBS at 50 C for 5 min then diluted in TBS so that the virus concentration was 2 ug/ml and the SDS concentration was 0.006%. Each of these virus solutions was added to the plates (50 ul/well) and incubated for 1-1.5 hr at room temperature. Following a 30 min blocking step of 1% bovine serum albumin (BSA) in TBS and one wash, monoclonal antibodies, diluted in TBS at concentrations from 20 ug/ml to 0.0064 ug/ml, were incubated in the wells (120 ul/well) at 4 C for 15 hr. The plates were then washed three times. The secondary antibody was alkaline phosphatase-labeled goat anti-mouse immunoglobulins (Kirkegaard & Perry, Gaithersburg, MD) diluted to approximately 5 ug/ml in TBS containing 0.1% BSA and incubated in the plates (50 ul/well) for 2.5 hr at room temperature. The plates were washed five times, and p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added (150 ul/well). Color development was observed and after one hour the plates were read on a Titertek Multiskan reader at an absorbance of 405 nm. The Multiskan was blanked against a row of wells that received all reagents except virus.

In indirect ELISA, virus was coated directly on the plates. Three different virus solutions were used. For untreated virus,

newly thawed virus was diluted to 2 ug/ml in TBS. For alkali treatment, virus was diluted to 2 ug/ml in 0.1 M carbonate pH 9.6 buffer. For SDS-dissociation, the virus was first incubated in a solution of 0.2% SDS in TBS at 60 C for 5 min and then diluted in TBS so that the virus concentration was 2 ug/ml and the SDS concentration was 0.006%. Each of these virus solutions was incubated in the plates for 1-1.5 hr at room temperature. The rest of the assay was performed identical to DAS ELISA.

Polyacrylamide gel electrophoesis, Western blotting and proteolysis

Purified virus was diluted in sample buffer (0.062 M Tris-HCl pH 6.8 containing 15% glycerol, 2% SDS and 5% 2-mercaptoethanol) heated at 100C for 5 min and applied (1-2 ug/lane) onto a gel composed of 12% acrylamide separating gel (0.375 M Tris-HCl, pH 8.8 + 0.1% SDS) with a 5% acrylamide stacking gel (0.125 M Tris-HCl, pH 6.8 + 0.1% SDS) (Laemmli, 1970). Electrode buffer was 0.025 M Tris, 0.192 M glycine pH 8.3 containing 0.1% SDS. Molecular weight markers were from Sigma Chemical Co., St. Louis, MO. Bands were visualized by direct Coomassie blue staining or immunological analysis with monoclonal antibodies. Transfer of proteins from gel to nitrocellulose membrane was accomplished by capillary blotting using 0.02 M Tris, 0.15 M NaCl, pH 7.5 (TBS). The blots were blocked for one hr in 2% BSA in TBS and incubated overnight at 4C in a solution of 2 ug monoclonal antibody per ml TBS containing 0.5% BSA. Following three successive five-minute washes in 0.1% BSA in TBS, the blots were incubated in a solution of rabbit anti-mouse immunoglobulins (Miles Scientific, Naperville, IL) at 2 ug/ml in TBS containing 0.5% BSA. After a 2 hr incubation and three successive washings, they were

incubated for 2 hr in goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) diluted 1:2000 in TBS containing 0.5% BSA. The blots were then washed and incubated for 5-10 min in TBS containing 0.5 mg/ml 4-chloro-1-napthol, 0.015% hydrogen peroxide.

In some experiments, purified virus (108 ug) was digested either with 1.1-5.8 ug trypsin TPCK (Worthington Biochemical Co., Freehold, NJ) in 388 ul of 0.05 M Tris pH 7.5 containing 0.38 mM NaCl and 0.05 mM $CaCl_2$, or with 11 ug proteinase K (Sigma Chemical Co., St. Louis, MO) in 398 ul of 0.05 M Tris pH 7.5. Following incubation at room temperature, reaction mixtures were diluted in sample buffer and the fragments separated by PAGE and blotted to nitrocellulose membrane for immunological analysis.

Neutralization of infectivity assay

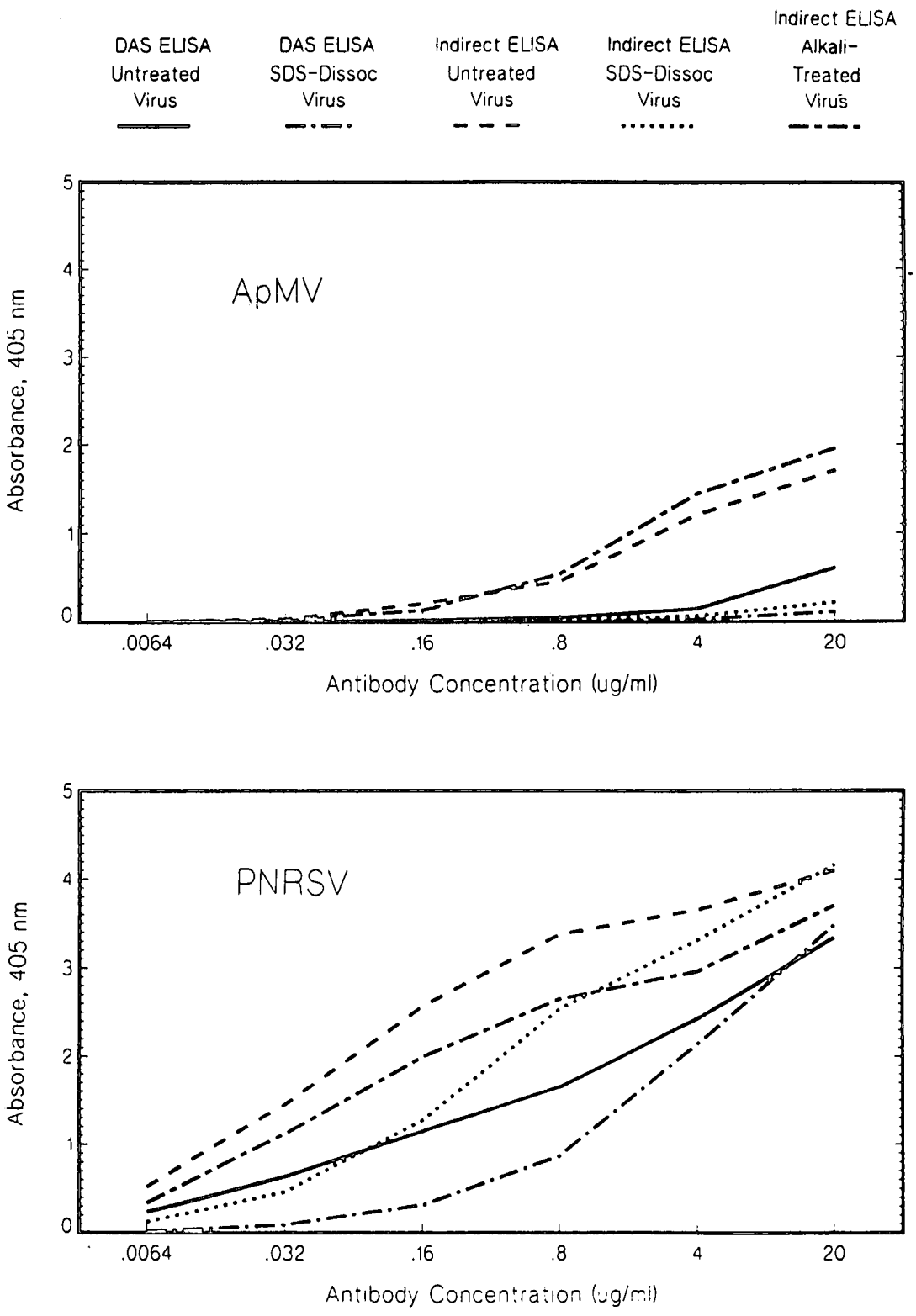
Purified PNRSV at a concentration of 10 ug/ml in 0.05 M Tris buffer pH 7.5 was incubated for 5.5 hr at 4C with various concentrations of monoclonal antibody. Cotton-tipped applicators were used to apply reaction mixtures onto carborundum-dusted cotyledons of Lemon cucumber. After 4 days, the number of lesions was recorded. Monoclonal antibodies 4, 5, 6 and 7 were tested, as well as antibody 8, which is a monoclonal antibody (CER 22A2H8) made to a member of caulimovirus, carnation etched ring virus, and is specific only to that virus (Hsu and Lawson, 1985).

RESULTS

To delineate the epitope specificity of the Mabs, the immunoreactivities were tested with untreated and SDS-dissociated virus on both rabbit globulin-coated (DAS ELISA) and virus-coated plates (indirect ELISA) (Appendix B; Jordan, Aebig and Hsu, manuscript in preparation). One of the ELISA tests was virus coated in carbonate buffer pH 9.6, included for comparison since this was the standard ELISA used to screen for Mabs when the hybridomas were produced. Binding curves for each of the five different ELISA tests titrating the Mabs in five-fold dilutions beginning with 20 ug/ml were generated. Due to the large quantity of data, only binding curves for Mab 4 are presented here (Fig. 2). Mab 4 reacted with SDS-dissociated PNRSV but not SDS-dissociated ApMV. Since in indirect ELISA, virus adsorption to plastic disrupts the virus (Jordan, Aebig and Hsu, manuscript in preparation), the ELISA method which best identifies Mab reaction with intact virions is DAS ELISA in which rabbit antibodies bound to the plastic trap virus particles. Mab 4 reacted well with intact PNRSV and weakly with intact ApMV. For both PNRSV and ApMV, Mab 4 reacted with alkali-treated virus and virus adsorbed to plastic.

The immunoreactivities of the other six Mabs with both viruses were determined using the five ELISA tests (Appendix B; Jordan, Aebig and Hsu, manuscript in preparation). Generally, the ApMV-specific Mabs 1, 2 and 3 had stronger reactivities with virus in indirect ELISA than with virus in DAS ELISA and stronger reactivities with disrupted virus than with untreated virus. The

Fig. 2. Binding curves of monoclonal antibody 4 with apple mosaic virus (ApMV) and Prunus necrotic ringspot virus (PNRSV). The antibody was purified from ascitic fluid by ammonium sulfate precipitation and column chromatography and titrated in five different ELISA tests. In double antibody sandwich ELISA (DAS ELISA), rabbit anti-virus immunoglobulins at 2 ug/ml were coated onto polyvinyl chloride plates. Either purified virus or 0.2% SDS-dissociated virus, at 2 ug/ml, was added. In indirect ELISA purified virus at 2 ug/ml was adsorbed directly onto the plates. The virus was either coated in Tris-buffered saline pH 7.5 (TBS), coated in carbonate buffer pH 9.6 or dissociated first in 0.2% SDS then coated in TBS. In all ELISA tests the subsequent steps were blocking with 1% BSA, followed by the addition of purified antibody at various concentrations. The detecting antibody was alkaline phosphatase-labeled goat anti-mouse immunoglobulins.


DAS ELISA Untreated Virus
DAS ELISA SDS-Dissoc Virus
Indirect ELISA Untreated Virus
Indirect ELISA SDS-Dissoc Virus
Indirect ELISA Alkali-Treated Virus
ApMV
Absorbance, 405 nm
0
1
2
3
4
5
.0064
.032
.16
.8
4
20
Antibody Concentration (ug/ml)
PNRSV
Absorbance, 405 nm
0
1
2
3
4
5
.0064
.032
.16
.8
4
20
Antibody Concentration (ug/ml)

PNRSV-specific Mabs 5, 6 and 7 reacted stronger with untreated than with disrupted virus.

From these and additional immunoblot assays (see Introduction), the epitopes on ApMV and PNRSV can be broadly characterized as conformation independent or dependent, and either external, hidden or partially hidden (Table II).

As previously mentioned, Mabs 1, 2 and 3 reacted with SDS, 2-ME-treated ApMV, and Mab 4 reacted with SDS, 2-ME-treated PNRSV in dot-blot assays. Thus, these Mabs were selected for use as probes in Western blot analysis of virus protein.

In experiments to verify that the antibodies react with viral coat protein, virus was dissociated and denatured in 2% SDS, 5% 2-ME sample buffer and the proteins separated by PAGE. The bands were visualized by Coomassie blue staining. The ApMV sample separated into a dense 26,000 dalton coat protein band and several minor bands. One was about 50,000 daltons, which could be a dimer of the coat protein, and the others were below 26,000 daltons which could be degradation products. The PNRSV sample also yielded a 26,000-27,000 coat protein band and a 50,000 dalton band, the latter which was not always visible especially if less than 1 ug virus was applied per lane.

Using Western blot techniques, viral protein was transferred from gels to nitrocellulose membrane and probed with the Mabs (Figs. 3 and 4). Mabs 1, 2 and 3 reacted with the coat protein of ApMV, although Mab 2 reacted very weakly. In addition, Mab 1 reacted with the dimer and the other minor bands. Mab 4 did not react with ApMV coat protein, but it did with PNRSV coat protein and also weakly

Table II. Summary of epitope characteristics.

	ApMV	PNRSV
Mabs 1, 2 and 3	conformation independent hidden epitopes	no reactivity
Mab 4	conformation dependent partially hidden epitope	conformation independent external eptitope
Mabs 5, 6 and 7	no reactivity	conformation dependent external epitopes

Fig. 3. Western blot of apple mosiac virus protein detection with monoclonal antibodies. Purified virus, diluted in sample buffer and heated at 100 C for 5 min, was applied (1-2 ug/lane) to a gel composed of 12% acrylamide separating gel with 5% stacking gel (Laemmli, 1970). Proteins were separated by electrophoresis and transferred from the gel to nitrocellulose membrane by capillary blotting. The blots were blocked in 2% bovine serum albumin and probed with monoclonal antibodies 1, 2, 3, 4 or 5 as indicated. Reactions were detected using rabbit anti-mouse immunoglobulins followed by horseradish peroxidase-labeled goat anti-rabbit immunoglobulins.

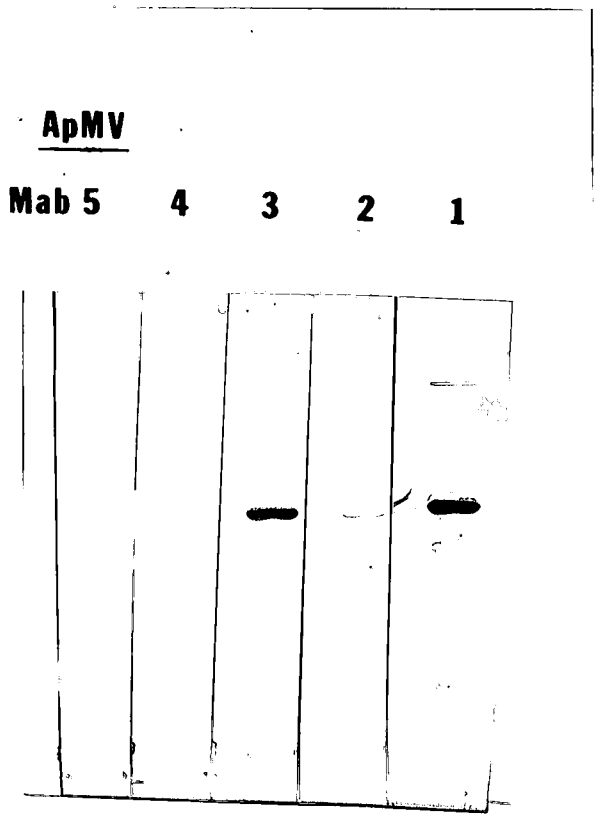
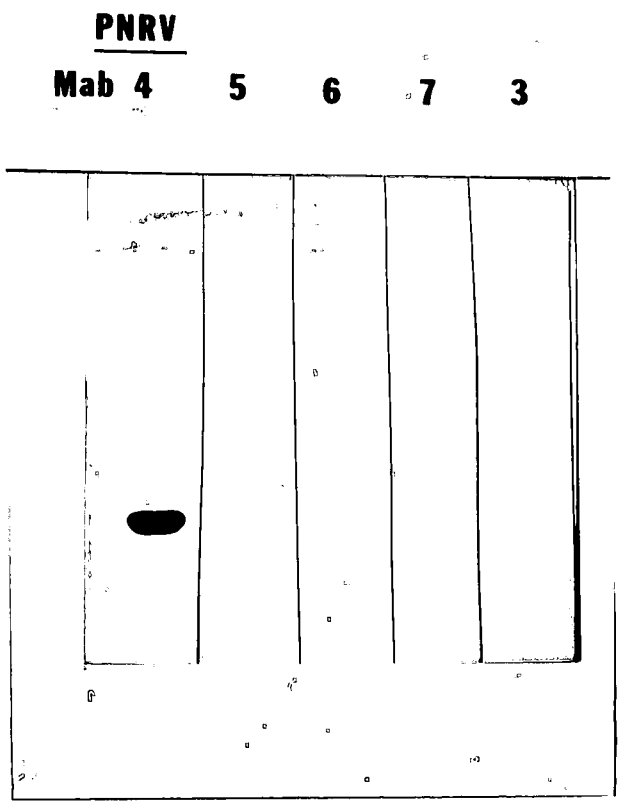

ApMV
Mab 5
4
3
2
1

Fig. 4. Western blot of Prunus necrotic ringspot virus protein detection with monoclonal antibodies. Purified virus, diluted in sample buffer and heated at 100 C for 5 min, was applied (1-2.5 ug/lane) to a gel composed of 12% acrylamide separating gel with 5% stacking gel (Laemmli, 1970). Proteins were separated by electrophoresis and transferred from the gel to nitrocellulose membrane by capillary blotting. The blots were blocked in 2% bovine serum albumin and probed with monoclonal antibodies 3, 4, 5, 6 or 7 as indicated. Reactions were detected using rabbit anti-mouse immunoglobulins followed by horseradish peroxidase-labeled goat anti-rabbit immunoglobulins.

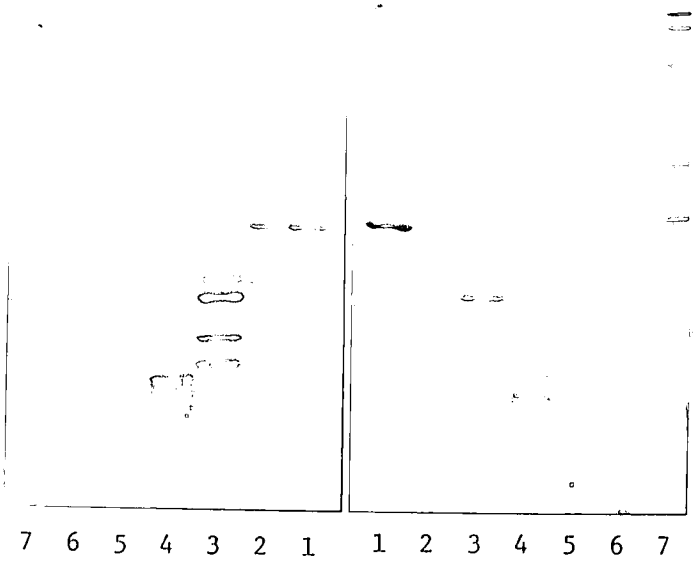

PNRV
Mab 4
5
6
7
3

with the dimer. Maps 5, 6 and 7 did not react with any of the protein bands in the PNRSV sample.

To further determine Mabs 1, 2 and 3 reactivity to ApMV and Mab 4 reactivity to PNRSV, the viruses were first protease digested, and the fragments separated by PAGE. It was hypothesized that the fragment containing the epitope could be identified using Western blot analysis. Fragments of the protein chain which did not contain the epitope would be visible in the Coomassie blue stain but not on the immunoblot. Protease digestion of ApMV did yield fragments, but on the blots, the bands were so faint that it was difficult to discern which bands corresponded to those on the stained gels (results not shown). The ApMV samples also often had degradation products so for subsequent studies efforts were concentrated on PNRSV only.

Intact PNRSV particles were digested with either trypsin TPCK, or proteinase K, diluted in sample buffer and separated by PAGE (Fig. 5). Direct Coomassie blue staining of the gels showed that a 30 min digestion with trypsin yielded two distinct proteolytic fragments, a 60 min digestion with trypsin yielded six fragments and a 10 min digestion with proteinase K yielded four fragments. In the latter two cases, a faint haze of low molecular weight protein materials was observed but resolution was very poor. The six tryptic fragments ranged from 17,000 to 24,500 daltons and each one was visible in both the stained gel and the blot. This indicated that the epitope recognized by Mab 4 was present on each of the fragments. However, the intensity of color of the blotted bands did not decrease with decreasing molecular weight. This

Fig. 5. Western blot analysis of proteolytic enzyme digests of Prunus necrotic ringspot virus coat protein. Purified virus was either untreated (lane 1), digested with trypsin for 30 min (lane 2), digested with trypsin for 60 min (lane 3), or digested with proteinase K for 10 min (lane 4) then heated in sample buffer at 100 C for 5 min. Samples were loaded (1-2 ug/lane) onto a gel composed of 12% acrylamide separating gel with 5% acrylamide stacking gel (Laemmli, 1970). Proteins were separated in the gel by electrophoresis and the bands were visualized by direct Coomassie blue staining (right side) or immunological analysis with monoclonal antibody 4 (left side). Transfer of proteins from gel to nitrocellulose membrane was accomplished by capillary blotting. The blots were blocked in 2% bovine serum albumin and probed with monoclonal antibody 4. Reactions were detected using rabbit anti-mouse immunoglobulins followed by horseradish peroxidase-labeled goat anti-rabbit immunoglobulins. Lanes 5 and 6 contained trypsin and proteinase K, respectively, without virus. Lane 7 was molecular weight standards in decreasing order, B-galactosidase (116,000 daltons), phosphorylase B (97,400 daltons), bovine albumin (66,000 daltons), ovalbumin (45,000 daltons), glyceraldehyde-3-phosphate dehydrogenase (36,000 daltons), carbonic anhydrase (29,000 daltons), trypsinogen (24,000 daltons), soybean trypsin inhibitor (20,100 daltons), and 2-lactalbumin (14,200 daltons).


7 6 5 4 3 2 1
1 2 3 4 5 6 7

implied that there were not multiple epitopes on each fragment. Putatively, proteolysis removed relatively small polypeptide portions on one or both ends of the main chain. The epitope, present once or in just a few copies, was retained on the large fragment that was resolved in the gels.

Additional experiments were performed in which PNRSV was digested with proteinase K or trypsin, either with or without 0.1% SDS in the reaction mixture. SDS disrupts virus structure and should expose additional cleavage sites. These digests were run in 5-25% polyacrylamide gradient gels to try to resolve any lower molecular weight peptide fragments. Digestion with either enzyme in the absence of SDS resulted in an expected banding pattern with no additional bands at lower molecular weights (results not shown). Digestion in the presence of SDS resulted in different banding patterns, but due to "smiling" and wide hazy bands, results were difficult to interpret.

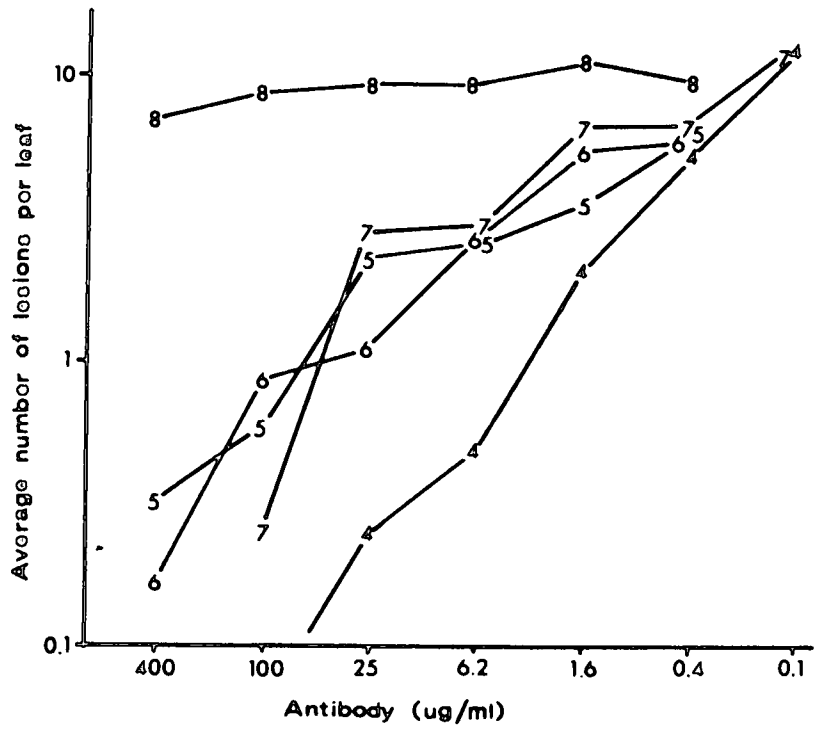
PNRSV was also treated with other enzymes, protease V8 and proline-specific endopeptidase, however no digestion occurred. In order to expose potential cleavage sites, the virus was first treated with 0.1% SDS and then incubated with enzyme. Still no fragments were visible on the stained gel (results not shown).

In one additional experiment, PNRSV was first incubated with Mab 4 (weight to weight ratio of 1:2.5) and the antigen-antibody complex was treated with trypsin or proteinase K. As hypothesized by Jemmerson and Paterson (1986), proteolysis of an epitope would be reduced if an antibody protected that region. Fragments generated by enzyme treatment of PNRSV-Mab4 complex were separated on

polyacrylamide gels and visualized by Coomassie blue staining (results not shown). Mab 4 did not protect the virus from digestion by proteinase K or trypsin. Mab 4 was digested with proteinase K but not with trypsin.

To determine if Mabs might affect the biological properties of PNRSV, neutralization of infectivity assays were performed (Fig. 6). Purified PNRSV was incubated with various concentrations of either Mab 4, 5, 6 or 7. One other antibody, Mab 8 was also included as a negative control. Mab 8 was produced to a member of caulimovirus, carnation etched ring virus, and is specific only to that virus (Hsu and Lawson, 1985). The figure shows that Mab 8 did not neutralize virus at any concentration tested. Mabs 4, 5, 6 and 7 all neutralized infectivity, Mab 4 to the greatest extent.

Fig. 6. Neutralization of Prunus necrotic ringspot virus infectivity. Purified PNRSV at a concentration of 10 ug/ml in 0.05M Tris buffer pH 7.5 was incubated for 5.5 hours at 4 C with various concentrations of monoclonal antibody. Cotton-tipped applicators were used to apply reaction mixtures onto carborundum-dusted cotyledons of Lemon cucumber. After 4 days, the number of lesions was recorded. Each point represents the average number of lesions on 12 leaves. (The average number of lesions that developed on each of 36 leaves following inoculation with virus at 10 ug/ml containing no antibody was 8.8). Monoclonal antibodies 4, 5, 6 and 7 were tested as indicated. Antibody 8 was a monoclonal antibody made to a member of caulimovirus, carnation etched ring virus, and is specific only to that virus (Hsu and Lawson, 1985).


Average number of lesions per leaf
10
1
0.1
400
100
25
6.2
1.6
0.4
0.1
Antibody (ug/ml)

DISCUSSION

It is interesting to note that Mab 4 binds to PNRSV coat protein but not ApMV coat protein. In ELISA tests, Mab 4 does bind to ApMV virus coated on the plates but not to virus dissociated with SDS or denatured with 2-mercaptoethanol and only weakly to intact virions (some results unpublished). The Mab 4 epitope on ApMV is conformation dependent, perhaps hidden between subunits. The site becomes exposed when the virus is partially disrupted but destroyed when the subunits are separated with SDS.

The Mab 4 epitope on PNRSV is, however, present on both intact virions and dissociated and denatured protein. PNRSV and ApMV are serologically related as determined using polyclonal sera, and the Mab 4 epitope is one of the common antigenic determinants. However, the epitope is very different on each virus, as determined by the binding characteristics with Mab 4.

Attempts to isolate Mab 4 epitope on a fragment of the PNRSV protein were unsuccessful. Proteolysis of the protein yielded bands on Coomassie blue stained polyacrylamide gels, and when Western blots were probed with Mab 4, all those bands were detected. Since 12% acrylamide gels do not resolve proteins of less than about 11,000 daltons, small peptides resulting from proteolytic digestion of PNRSV protein cannot be detected. Even though the 5-25% gradient gels also yielded no lower molecular weight bands, the following observations can be made. There are several cleavage sites recognized by trypsin. One or two are initially accessible and the others become accessible only after additional incubation. After 30 min of incubation, three fragments from 24,000 to 22,000 daltons are

generated. Since the molecular weight of full length PNRSV protein is 27,000 daltons, the molecular weights of small fragments cleaved off are only several thousand daltons. With time, the cleavage site that results in the generation of the smallest of those three fragments becomes more accessible to trypsin, since more of that fragment is observed after 60 minutes of incubation. Additional sites become available to the enzyme, and other bands from 19,000 to 17,000 daltons appear in the gel and on the blot. The action of trypsin on the virus could be the sequential recognition and cleavage of sites from one or both ends of the protein, which become accessible as virion structure is gradually degraded. There are no cleavage sites available on the intact virion which result in the generation of a fragment larger than 11,000 daltons which does not contain the epitope. Rather, all resolved bands contained the epitope. Since the color intensity of the bands does not decrease with decreasing molecular weight, there are probably not multiple Mab 4 epitopes on the polypeptide chain, just one or a few. If there were multiple epitopes which were sequentially cleaved off by trypsin, the remaining fragments in the gel would be devoid of those sites, so the color intensity would decrease with decreasing fragment length.

The enzymatic degradation of virions is an interesting topic since Bol et al. (1974) found that trypsin treatment of AMV particles removed 25 amino acids from the N-terminus of the coat protein. Since the N-terminus binds to the RNA in the interior of the virions, it is necessary that trypsin molecules move into the particle to reach arginine and lysine residues within (Jaspars,

1985). Cleavage at these sites interferes with protein-RNA interactions thereby destabilizing the particle.

It is an odd phenomenon that the 22,000 dalton trypsin molecule moves through holes in the capsid considering a function of coat protein is to protect viral RNA. Many viruses have been shown to undergo in situ degradation by trypsin, as found with PNRSV in the present study (cowpea chlorotic mottle virus, Tremaine et al., 1972; potyviruses, Hiebert et al., 1984; AMV and brome mosaic virus, Bol et al., 1974; potato virus X, Koenig and Torrance, 1986). It is interesting that Mab binding to PNRSV virus particles does not protect them from proteolytic enzymes. Protease molecules apparently bypass the antibodies coating the surface of the virion and locate cleavage sites which may be internal.

The studies with Mab 4 reactivity against PNRSV protein and the neutralization of virus infectivity suggest that Mab 4 binds to an epitope that is located in a region of the coat protein necessary for infection. Information on the nature of PNRSV attachment and penetration of cells is unavailable. Currently, it is not known if the mechanism of Mab 4 neutralization of PNRSV infectivity involves an event inside or outside the cell membrane. Hypotheses for the action of Mab 4 binding to the virus include: 1) blocking viral anti-receptors, thereby preventing entry of the virus into the cell, 2) preventing uncoating of the virus once inside the cell, or 3) interfering with coat protein-RNA interactions necessary for RNA infection. The second and third hypotheses are interesting based on what is known about ilarvirus coat protein dependent initiation of infection. The function of the coat protein is probably not

penetration into a cell since RNA alone is infectious. Its function, therefore, is more likely an intracellular one.

Further discussion on the possible function of coat protein first requires a brief summary of what is known about AMV (from Jaspars, 1985). One property of AMV coat protein is the binding of its N-terminal region to the 3' end of AMV RNA. All four AMV RNAs and TSV RNA 3 have 3' end coat protein binding sites. In addition, these RNAs can have several coat protein binding sites throughout the RNA sequence. In some instances heterologous coat protein binds to these sites (Zuidema et al., 1984). No minus strand RNAs have been found to possess coat protein binding sites. Based on what is known about coat protein dependent initiation of infection, it is likely that the binding sites, when bound to an activating coat protein, provide the necessary biological activity to start infection. In AMV infection with the three largest RNAs, there is evidence that all must be bound to coat protein for infection to take place. In infection with the four RNAs in the absence of coat protein, naked RNA 4 is translated to yield coat protein which then binds to the other three, and infection proceeds. Clearly coat protein is not required for the initial translation of RNA 4 or in vitro translation of RNAs 1, 2 and 3. The function of the coat protein then would appear to be the formation of the nucleoprotein structure necessary for replication of genomic RNA, i.e., minus-strand synthesis. The nucleoprotein, in combination with the replicating enzyme and possibly additional host proteins, would comprise the replication complex.

In PNRSV, a possible effect of Mab 4 binding to the

virus could be preventing uncoating of the virus inside the cell and/or interfering with coat protein-RNA interaction. Mab 4 could stabilize capsid structure so that RNA is not released, or it could interfere with protein conformational structure needed to bind RNA and combine with other factors to form the replication complex.

Monoclonal antibody 4 may be useful in studies to elucidate the replication strategy of the ilarviruses. Experimentation would probably require a protoplast system to culture PNRSV since manipulations with whole plants can be difficult. Time course studies on the location of labeled antibodies and virions in the protoplasts may provide information on antibody-antigen interaction during the infection cycle. Reconstitution studies of coat protein, RNA and antibody and isolation of the complexes on polyacrylamide gels may detect inhibition of coat protein-RNA interaction by the antibody. Photochemical cross-linking of RNA and proteins in infected cells may identify proteins required at various times during the replication cycle. This information would provide insight into the exact role of coat protein in activating the ilarvirus genome.

SUMMARY

1. The immunoreactivities of the monoclonal antibodies were assayed in various double antibody sandwich and indirect ELISA tests, and the locations of the epitopes on the virion were determined. Monoclonal antibodies 1, 2 and 3 bound to conformation independent, hidden epitopes on ApMV. Monoclonal antibody 4 bound to a conformation dependent, partially hidden epitope on ApMV and a conformation independent, external epitope on PNRSV. Monoclonal antibodies 5, 6 and 7 bound to conformation dependent external epitopes.

2. In Western blot analysis, Mabs 1, 2 and 3, but not Mab 4, reacted with ApMV coat protein. Mab 4 reacted with PNRSV coat protein, whereas, Mabs 5, 6 and 7 did not.

3. Fragments of PNRSV coat protein were generated by proteolysis, and, in Western blots, all were bound by Mab 4.

4. In neutralization of infectivity studies, Mabs 4, 5, 6 and 7 blocked PNSRV infectivity. It was previously shown that Mabs 5, 6 and 7, but not Mab 4, precipitated PNRSV in Ouchterlony double diffusion tests.

CONCLUSIONS

Monoclonal antibodies are useful probes for studying virus structure and biological properties due to the specificity of the reaction. Studies of the ilarviruses using Mabs 1-7 has yielded more information on epitopes and biological functions of coat protein than could be obtained using polyclonal sera. In various ELISA tests, whole virions or SDS-dissociated virions were assayed for reactivity with the Mabs. Based on these and additional dot-blot assays, the epitopes to which these Mabs bind were characterized as either conformation independent or dependent and either external, hidden or partially hidden. Using Western blot techniques, it was shown that some of the Mabs bind to viral coat protein.

It was hypothesized that the fragment of protease-digested coat protein that contained the epitope could be identified using Western blot analysis. Fragments of PNRSV coat protein were resolved on polyacrylamide gels, and all were found to bind Mab 4 on the blots, thus a specific fragment containing the epitope could not be identified. The epitope is likely to occur in just one or a few copies on the polypeptide chain. The Mab 4 epitope is located in a region of the coat protein necessary for infection since the antibody blocked infection in neutralization of infectivity tests. It is hypothesized that Mab 4 prevents uncoating of the virus or interferes with coat protein-RNA interactions necessary for the infection to proceed. The requirement of coat protein, or the subgenomic RNA which codes for coat protein, in the initiation of infection has been established for some ilarviruses and is likely to

be characteristic of PNRSV also.

Assuming the PNRSV replication system is analagous with AMV replication, a function of coat protein could be the formation of a nucleoprotein structure necessary for replication of viral RNA. Possible effects of Mab 4 are stabilizing the virion so RNA is not released or interfering with coat protein conformational structure needed to bind RNA and form the replication complex.

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APPENDIX A. Determination of affinity constants

In assays which compare antibodies of varying affinities, results can be influenced and distorted by the differential in degrees of binding (Nimmo, et al., 1984; Lew, 1984). It is therefore important to be aware of the relative affinities of the antibodies for consideration when interpreting results. The affinity constants (dissociation constants, K_D) of some of the monoclonal antibodies were determined by incubating antigen and antibody to equilibrium in solution and measuring the amount of unbound antibody by indirect ELISA (Friguet et al., 1985).

Methods

First, polyvinyl chloride plates were coated with 1% BSA in TBS for 3 hr at room temperature. Appropriate concentrations of purified virus and Mabs (determined previously by ELISA) were incubated in the coated wells. Two-fold serial dilutions of virus were made starting at either 81 ug/ml or 2.5 ug/ml. Mabs (from $AmSO_4$ precipitated ascitic fluid) were added to yield final concentrations of either 0.025, 0.05, 0.25, or 0.5 ug/ml. The plates were incubated overnight at 4 C. Next the contents of the wells (120 ul) were transferred to plates previously coated with antigen and blocked, and the amount of unbound antibody was determined by indirect ELISA (previously described).

The plates that were used for reacting the antigen and antibody in solution were assayed for the presence of antigen adsorbed to the wells using alkaline phosphatase-labeled rabbit anti-virus immunoglobulin and for antibody adsorbed to the wells using alkaline phosphatase-labeled goat anti-mouse immunoglobulins.

Conditions applied in these assays were similar to those in indirect ELISA (previously described).

Results

Calculations were made assuming the molecular weight of viral subunit was 29,000 daltons and the molecular weight of antibody was 150,000 daltons. Absorbance readings were recorded as the average of duplicate wells. The mathematical equation used to correlate absorbance with the fraction of bound antibody (v) was:

$$(A_o - A)/A_o = v \qquad (1)$$

where A_o is the absorbance measured for antibody alone and A is the absorbance measured for antibody incubated with various concentrations of antigen (Friguet et al., 1985). Total antigen and total antibody concentrations were related to the fraction of bound antibody, v, by the equation

$$a = (a_o - i_o)(v) \qquad (2)$$

where a is the calculated concentration of free antigen, a_o is the total concentration of antigen, and i_o is the total concentration of antibody. Next the expression v/a was calculated and Scatchard plots were drawn from linear regressions (Fig. 7). Dissociation constants were determined by two methods. One was calculated as negative the reciprocal of the slope value from the Scatchard plot. The second method was the equation $K_D=(1-v)/(v/a)$ using values obtained from the experimental data used to generate the Scatchard plots. Table III shows the dissociation constants and the relative affinity of the antibodies.

Discussion

Computations were also made using the molecular weight of the

Fig. 7. Linear regressions analysis of Scatchard plots of the binding of antibodies 1 and 4 to ApMV and the binding of antibodies 4, 5 and 7 to PNRSV. Absorbance values were obtained from indirect ELISA. v is the fraction of bound antibody and v/a is that fraction divided by the concentration of free antigen.

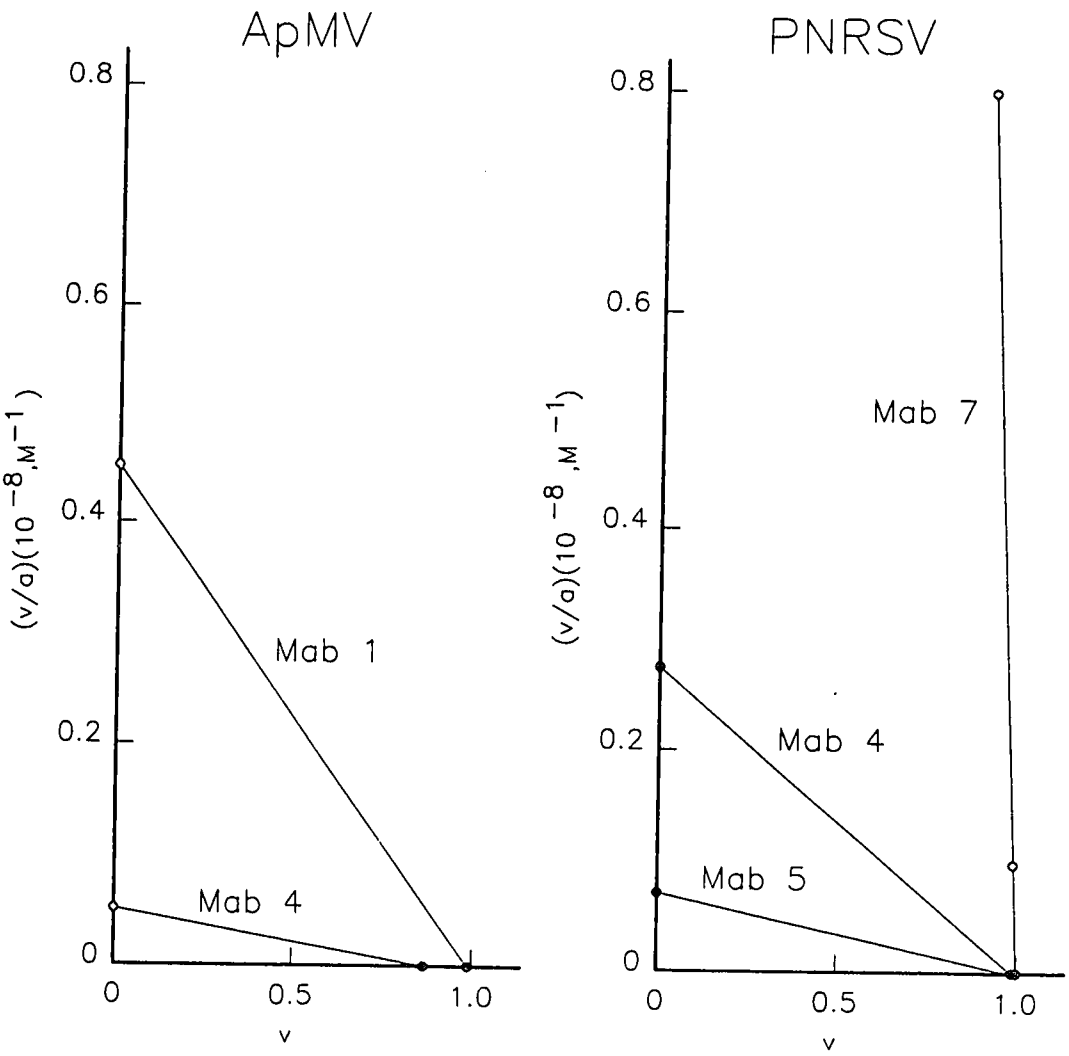
ApMV
PNRSV
0.8
0.6
0.4
0.2
0
0.5
1.0
v
(v/a)(10 $^{-8}$,M $^{-1}$)
Mab 1
Mab 4
Mab 7
Mab 5

TABLE III. Dissociation constants of antigen-bound antibody

	K_D		
	Method 1[a]	Method 2[b]	Relative Affinity
ApMV and Mab 1	2.3×10^{-8}M	2.8×10^{-8}M	1 > 4
ApMV and Mab 4	17×10^{-8}M	45×10^{-8}M	
PNRSV and Mab 4	3.5×10^{-8}M	7×10^{-8}M	
PNRSV and Mab 5	15×10^{-8}M	11×10^{-8}M	7 > 4 > 5
PNRSV and Mab 7	$.07 \times 10^{-8}$M	$.08 \times 10^{-8}$M	

[a] K_D = 1/-slope

[b] K_D = (1-v)/(v/a)

whole virus, 5,300,000 daltons, rather than subunit molecular weight (results not shown). If the molecular weight of the antigen is very large, the molar concentration, a_o, is very small, and from equations (1) and (2) the values for v/a are negative for high affinity antibodies and positive but disproportionately high for low affinity antibodies. The equations were developed for small haptens and must be adapted for macromolecules. Reducing the value of the molecular weight by using that of the subunit can be justified by assuming the antigen is multivalent; however, this introduces excessive error in the calculations. The equations therefore, cannot be used to determine the actual value for the dissociation constants. Rather, this technique may be an indication of relative affinity only, and the results do concur with the order of relative affinity previously determined from binding curves (Jordan and Aebig, 1985).

A disadvantage of this method in determining relative affinity is that large amounts of antigen are required to satisfy the condition of large antigen excess. The antibody concentration, predetermined by ELISA, was chosen which gave an absorbance value between 0.3 and 1.0. The concentration ranged from 0.025 to 0.5 ug/ml depending upon the antibody. To satisfy antigen excess, 81 ug virus per ml was needed to start the dilution series.

Some theoretical considerations which question the validity of using ELISA to determine actual affinity constants of antibody binding to virus particles follow. 1. Even though the soluble antigen and antibody are incubated to equilibrium, the mixture is no longer at an equilibrium state when transferred to plates coated

with antigen. If there is differential binding of the antibody to soluble versus plastic-bound antigen, then conditions in the tests are altered such that the equations no longer apply. 2. In BSA coated plates, there is still some antigen which binds at higher concentrations (results not shown). Above 20 ug/ml, antigen can be detected bound in the wells using enzyme-labeled rabbit anti-virus immunoglobulins even though antibody cannot be detected using enzyme-labeled goat anti-mouse immunoglobulins. Thus, some antigen is not in solution during the antibody-antigen incubation step.

As previously mentioned, the relative affinities of antibodies can influence the results of various assays. Jordan and Aebig (1985) found that it is important to consider the relative affinities of the antibodies when analyzing results from competitive inhibition assays.

Appendix B

Jordan, R. L., J. A. Aebig and H. T. Hsu. 1985. Reactivities of apple mosaic virus (ApMV)/Prunus necrotic ringspot virus (NRSV) monoclonal antibodies (McAbs) with ApMV, NRSV and other ilarviruses. Phytopathology 75:1353.

Seven McAbs made to ApMV and/or NRSV were evaluated in various direct and indirect ELISA and immmunodot-blot assays. Virus treatments included 0.2-2% sodium dodecyl sulfate with and without 5% mercaptoethanol, and pH 9.5/56°C. Generally, McAbs 1, 2, and 3 gave stronger reactivities with treated ApMV than with untreated virus. Conversely, McAbs 4, 5, and 6 reacted stronger with untreated ApMV. McAbs 5, 6 and 7 reacted strongest with untreated NRSV, and McAb 4 reacted well with both treated and untreated NRSV. In other indirect ELISA tests, McAbs 2, 3, and 6 were found to react with several other ilarviruses, specifically tobacco streak virus, citrus variegation virus, prune dwarf virus, as well as alfalfa mosaic virus. In Western blot analysis with intact coat protein (CP) subjunits and enzyme-digested CP subunit fragments, only McAbs 1, 2, and 3 react with ApMV and only McAb 4 reacts with NRSV.

Appendix C

Aebig, J. A., R. L. Jordan, and H. T. Hsu. 1986. Immunological and biological studies of Prunus necrotic ringspot ilarvirus using a mouse monoclonal antibody. Phytopathology 76:1132.

A monoclonal antibody which reacts with Prunus necrotic ringspot ilarvirus (PNRSV) was used in immunochemical studies and neutralization of infectivity assays. The antibody was able to detect the 27000 relative molecular weight coat protein of PNRSV in Western blot analysis. The monoclonal antibody also detected all polypeptide fragments that were generated after incubation of whole virus with proteolytic enqymes. In neutralization of infectivity studies, the antibody blocked virus infectivity although it did not precipitate the antigen in Ouchterlony double diffusion tests.

Appendix D

Immunochemical and biological properties of a mouse monoclonal antibody reactive to Prunus necrotic ringspot ilarvirus

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Running title: Ilarvirus monoclonal antibody

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Key words Ilarviruses, monoclonal antibody, neutralization, proteolytic fragments, Western blot.

Summary. A monoclonal antibody which reacts with Prunus necrotic ringspot ilarvirus (PNRSV) was used in immunochemical studies and neutralization of infectivity assays. This antibody was able to detect the 27000 relative molecular weight coat protein of PNRSV in Western blot analysis. The monoclonal antibody also detected all polypeptide fragments that were generated after incubation of whole virus with proteolytic enzymes. In neutralization of infectivity studies, the antibody blocked virus infectivity although it did not precipitate the antigen in agar gels by Ouchterlony double diffusion tests. Immune electron microscopy confirmed that the antibody coats virions but does not cause clumpimg. This study suggests the antibody may be a useful tool for investigating coat protein dependent initiation of infection.

Ilarviruses are a group of isometric labile viruses which frequently produce ringspot or line pattern symptoms in woody and herbaceous hosts (Francki, et al., 1985). Prunus necrotic ringspot virus (PNRSV) has the characteristic ilarvirus multicomponent system consisting of three different sedimenting nucleoprotein components and four different RNA species (Loesch and Fulton, 1975; Gonsalves and Fulton, 1977). RNA's 1, 2 and 3 are not infectious; however, when RNA 4 is added to the other three, infectivity is regained (Gonsalves and Fulton, 1977). Although not yet investigated for PNRSV, it has been shown for other ilarviruses and for alfalfa mosaic virus, that coat protein can be substituted for RNA 4 in activating RNA's 1, 2 and 3; and, in certain cases, the coat proteins are interchangeable as activators of RNA in initiating infection (Gonsalves and Garnsey, 1975; van Vloten-Doting, 1975). This property of coat protein dependent initiation of infection has been investigated through studies of coat protein - RNA interactions (Jaspars, 1985; Zuidema et al., 1984). In this report, we describe a potential tool for studying the infection process, a monoclonal antibody which reacts with PNRSV coat protein and neutralizes virus infectivity.

Biological and immunochemical properties of purified PNRSV, (ATCC PV 22; Fulton, 1959) were examined using monoclonal antibodies (Mabs) previously generated against a mixture of ilarviruses (Halk et al., 1984). Four of these antibodies, Mabs 4, 5, 6 and 7 (formerly NA70C9, NA49F8, N46E10 and N63F10, respectively, described by Halk et al., 1984) react with PNRSV in ELISA tests and dot-blot immunoassays (Jordan et al., 1985).

To further characterize antibody reactivity with PNRSV, dot blot immunoassays were performed using purified virus, 0.2% SDS treated virus, and

2% SDS, 5% 2-mercaptoethanol treated virus (Fig. 1). Mabs 5, 6 and 7 did not react to viruses which were exposed to the dissociating conditions of SDS and the reducing conditions of 2-mercaptoethanol suggesting those epitopes are conformation dependent (Fig. 1, some data not shown). Mab 4, however, reacted with SDS, 2-mercaptoethanol treated virus which indicates that the Mab 4 specific epitope is a conformation independent determinant. The results also indicated the usefulness of Mab 4 as a probe in Western blot analysis.

In experiments to verify the reactivity of Mab 4 with PNRSV coat protein, SDS-polyacrylamide gel electrophoresis of virus coat protein was performed. Using Western blot techniques, viral protein was transferred from polyacrylamide gel to nitrocellulose membrane and probed with Mab 4. Mab 4 reacted with the 27000 relative molecular weight (27K Mr) coat protein band and a minor 50K Mr band, which could be a dimer (results not shown). The 50K Mr band was not always visible, especially if less than 1 ug virus was applied in each well. As expected Mabs 5, 6 and 7 did not react with either the 27 K Mr or 50K Mr band.

Since Mab 4 reacted to reduced coat protein, it was hypothesized that the region on the polypeptide chain that contains the epitope could be identified by fragmenting the viral protein and using Western blot analysis to determine which fragment(s) contain the epitope. Whole virus was digested with either trypsin TPCK (Worthington Biochemical Co., Freehold, NJ) or proteinase K (Sigma Chemical Co., St. Louis, MO), diluted in sample buffer and separated by electrophoresis on polyacrylamide gels. Direct Coomassie blue staining of polyacrylamide gels (Fig. 2, right) showed the presence of at least (i) two distinct proteolytic fragments following 30 minutes of digestion with trypsin (lane 2); (ii) six distinct proteolytic fragments following 60 minutes of

digestion with trypsin (lane 3) and; (iii) four proteolytic fragments following ten minutes of digestion with proteinase K (lane 4). In the latter two cases, a faint haze of low molecular weight protein materials was observed, but resolution was very poor in 12% acrylamide gels. On immunoblots, Mab 4 reacted with all the proteolytic fragments since identical bands were observed on nitrocellulose membranes and Coomassie blue stained polyacrylamide gels (Fig. 2, left).

The results of tryptic enzyme digestion suggests that the epitope is present only once on the polypeptide. This can be deduced from the following observations. There are several cleavage sites recognized by trypsin. One or two of sites are initially accessible and the others became accessible only after additional incubation. Cleavage results in six polypeptide fragments with relative molecular weights ranging from 17K to 24.5K daltons each of which contain the epitope recognized by Mab 4. Because the intensity of color of the bands does not decrease with decreasing molecular weight, there are probably not multiple epitopes (repeating epitopes) on the polypeptide chain.

A third enzyme, proline-specific endopeptidase (Miles Laboratories) was also used to treat whole virus, however, no digestion occurred. In order to expose potential cleavage sites, the virus was first treated with 0.1% SDS and then incubated with enzyme. Still no fragments were visible by Coomassie blue staining after enzyme treatment (results not shown).

In tests to determine if reaction with monoclonal antibodies might affect the biological properties of the virus, neutralization of infectivity assays were performed. Purified PNRSV was incubated with various concentrations of monoclonal antibody and inoculated onto Lemon cucumber cotyledons. After 5 days, the number of lesions that developed was recorded. Figure 3 shows

results of neutralization of infectivity of PNRSV by Mabs 4, 5, 6 and 7 and also Mab 8 which is a monoclonal antibody made to a heterologous virus, carnation etch ring virus (Hsu and Lawson, 1985). Clearly, Mabs 4, 5, 6 and 7 all were able to neutralize virus infectivity.

The reactivity of of PNRSV with Mabs 4 and 7 was tested by electron microscopy to determine if precipitation and/or coating of the virions occurs with each antidody. Purified virus and monoclonal antibodies were mixed and incubated on grids, stained and observed under the electron microscope. Mab 7 produced clumping of the virions and many particle clusters were observed with an associated accumulation of the electron dense stain (Fig. 4a). Mab 4 did not produce clumping of the virions and many, but not all, particles were surrounded with the electron-dense stain and associated antibodies (Fig. 4b). PNRSV incubated with either monoclonal antibody specific to apple mosaic virus but not PNRSV (A63E10; Halk et al., 1984), or carnation etched ring virus-specific monoclonal antibody 8 (CER 22A2H8; Hsu and Lawson, 1985) showed no clumping or accumulation of electron-dense stain around the virions (Fig 4 c, d). Electron microscopy clearly showed that Mabs 7 and 4, but not the two heterologous antibodies, attach to the virions. It was previously shown that Mabs, 5, 6, and 7 reacted with PNRSV in Ouchterlony double diffusion tests and formed visible precipitates with the virus (Halk et al., 1984). Mab 4, however, did not precipitate the virus. Electron microscopy confirmed these results.

Mab 4, then, identifies a specific epitope, present once on each polypeptide chain. This region of the protein may play a role in initiating infection, since binding of the epitope with antibody neutralized virus infectivity. The mechanism for neutralization does not apparently involve

virus precipitation.

It was hypothesized that non-precipitating antibodies may be specific for antigenic sites inaccessible to antibody binding with intact virions (Halk, et. al, 1984). Results from present studies of neutralization of PNRSV infection and immune electron microscopy indicate otherwise for Mab 4. Double antibody sandwich ELISA tests also indicate that Mab 4 reacts with untreated virus (Jordan et al., 1985). Mab 4 defines an epitope that is accessible to the antibody, although it does not precipitate the virus possibly because the distance between epitopes on different virions is too great or because of an inflexible hinge.

These studies on the immunoreactivity of Mab 4 with PNRSV and the effect of the antibody on virus infectivity suggest that Mab 4 binds to an epitope that is located in a region of the coat protein polypeptide necessary for infection. The binding of Mab 4 to the virus could possibly prevent uncoating of the virus, prevent entry of the virus into the cell, or perhaps interfere with coat protein-RNA interactions necessary for RNA infection. Mab 4 is an antibody reactive in ELISA to at least 15 isolates of apple mosaic virus and PNRSV tested including Danish plum line pattern virus (ATCC PV34) (Halk, et al., 1984). Mab 4 may provide a useful tool in studying coat protein dependent initiation of infection and viral replication of PNRSV and other ilarviruses.

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FIGURE CAPTIONS

Fig. 1. Dot-blot immunoassays of Prunus necrotic ringspot virus. Preparations of purified virus, in 0.05 M Tris pH 7.5, were either untreated, treated with 0.2% sodium dodecyl sulfate (SDS) at room temperature for 30 min., then at 60C for 5 min., or treated with 2% SDS, 5% 2-mercaptoethanol (2-ME) at 100C for 3 min. The samples were diluted in 0.05 M Tris pH 7.5 so that SDS concentrations were less than 0.11% and 2-ME concentrations were less than 0.19%. The 97 ul samples were applied onto 0.2 um pore size nitrocellulose membranes using a Minifold apparatus (Schleicher & Schuell, Inc., Keene, NH). The blots were air dried, blocked for 1 hr in 2% bovine serum albumin (BSA) in 0.02 M Tris, 0.15 M NaCl pH 7.5 (TBS) and incubated overnight at 4C in monoclonal antibody 4(a) or 7(b) at 1 ug/ml in TBS containing 0.5% BSA (TBS-BSA). Following three successive ten minute washes in TBS-BSA the blots were incubated overnight at 4C in rabbit anti-mouse affinity purified IgG + IgM + IgA (Zymed Laboratories, Inc., San Francisco, CA). Following washes in TBS-BSA, the blots were incubated overnight at 4C in goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) diluted 1:3000 in TBS-BSA. The blots were then washed and incubated for 5-10 min in TBS containing 0.5 mg/ml 4-chloro-1-napthol, 0.015% hydrogen peroxide.

Fig. 2. Western blot analysis of proteolytic enzyme digests of Prunus necrotic ringspot virus coat protein. Purified virus (108 ug) was digested either with 1.1-5.8 ug trypsin TPCK (Worthington Biochemical Col., Freehold, NJ) in 388 ul 0.05 M Tris pH 7.5 containing 0.38 mM NaCl and 0.05 mM $CaCl_2$, or with 11 ug proteinase K (Sigma Chemical Co., St. Louis, MO) in 398 ul 0.05 M Tris pH 7.5. Following incubation (see below) reaction mixtures were diluted in sample buffer (0.0620M Tris-HCl pH 6.8 containing 15% glycerol, 2% SDS and 5% 2-mercaptoethanol) heated at 100C, 5 min., and applied (1-2 ug/lane) onto a gel composed of 12% acrylamide separating gel (0.375 M Tris-HCl, pH 8.8 + 0.1% SDS) with a 5% acrylamide stacking gel (0.125 M Tris-HCl, pH 6.8 + 0.1% SDS) (Laemmli, 1970). Electrode buffer was 0.025 M Tris, 0.192 M glycine pH 8.3 containing 0.1% SDS. Bands were visualized by a) direct Coomassie blue staining (right side) or immunological analysis with Mab 4 (left side). Transfer of proteins from gel to nitrocellulose membrane was accomplished by capillary blotting using 0.02 M Tris, 0.15 M NaCl, pH 7.5 (TBS). The blots were blocked for one hour in 2% BSA in TBS and incubated overnight at 4C in a solution of 2 ug monoclonal antibody per ml TBS containing 0.5% BSA. Following three successive five-minute washes in 0.1% BSA in TBS, the blots were incubated in a solution of rabbit anti-mouse immunoglobulins (Miles Scientific, Naperville, IL) at 2 ug/ml TBS containing 0.5% BSA. After a 2 hour incubation and three successive washings, they were incubated for 2 hours in goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) diluted 1:2000 in TBS containing 0.5% BSA. The blots were then washed and incubated for 5-10 min. in TBS containing 0.5 mg/ml 4-chloro-1-napthol, 0.015% hydrogen peroxide. Lanes 1, 2, 3 and 4 are, respectively, undigested viral coat

protein, digestion with trypsin at room temperature for 30 min., digestion with trypsin at room temperature for 60 min. and digestion with proteinase K at room temperature for 10 min. Lanes 5 and 6 contain trypsin and Proteinase K, respectively, without virus. Lanes 7 is molecular weight standards, in decreasing order, B-galactosidase, phosphorylase B, bovine albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, and 2-lactalbumin (Sigma Chemical Co., St. Louis, MO).

Fig. 3. Neutralization of Prunus necrotic ringspot virus infectivity. Purified PNRSV at a concentration of 10 ug/ml in 0.05M Tris buffer pH 7.5 was incubated for 5.5 hours at 4C with various concentrations of monoclonal antibody. Cotton-tipped applicators were used to apply reaction mixtures onto carborundum-dusted cotyledons of Lemon cucumber. After 4 days, the number of lesions was recorded. Each point represents the average number of lesions on 12 leaves. (The average number of lesions that developed on each of 36 leaves following inoculation with virus at 10 ug/ml containing no antibody was 8.8). Monoclonal antibodies 4, 5, 6 and 7 were tested as indicated. Antibody 8 is a monoclonal antibody (CER 22A2H8) made to a heterologous virus, carnation etched ring virus (Hsu and Lawson, 1985).

Fig. 4. Immune electron microscopy of monoclonal antibody reactivity to Prunus necrotic ringspot virus. A 10 ul drop of purified virus at a concentration of 7.0 ng/ml in 0.01M Tris, pH 7.4 was placed on a carbon-coated Formvar grid, and a 10ul drop of the respective monoclonal antibody at 5 ug/ml was added and mixed with a pasteur pipette. The reactants were incubated for 30 minutes and stained with fresh uranyl formate. Virus was incubated with

either (a) monoclonal antibody 7, (b) monoclonal antibody 4, (c) a monoclonal antibody specific to apple mosaic virus but not Prunus necrotic ringspot virus (A63E10; Halk et al., 1984). or (d) carnation etched ring virus-specific monoclonal antibody 8 (CER 22A2H8; Hsu and Lawson, 1985). All antibody preparations were diluted in 0.01M Tris, pH 7.4. Bar= 100 nanometers.

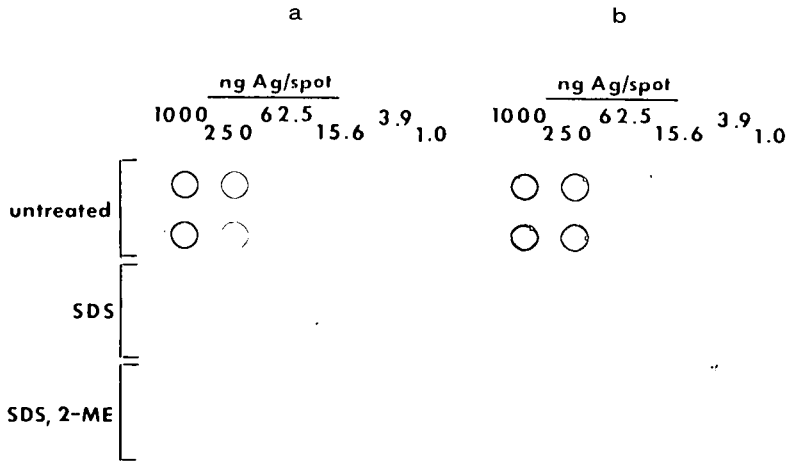

a
b
ng Ag/spot
1000 250 62.5 15.6 3.9 1.0
ng Ag/spot
1000 250 62.5 15.6 3.9 1.0
untreated
SDS
SDS, 2-ME

Fig. 2

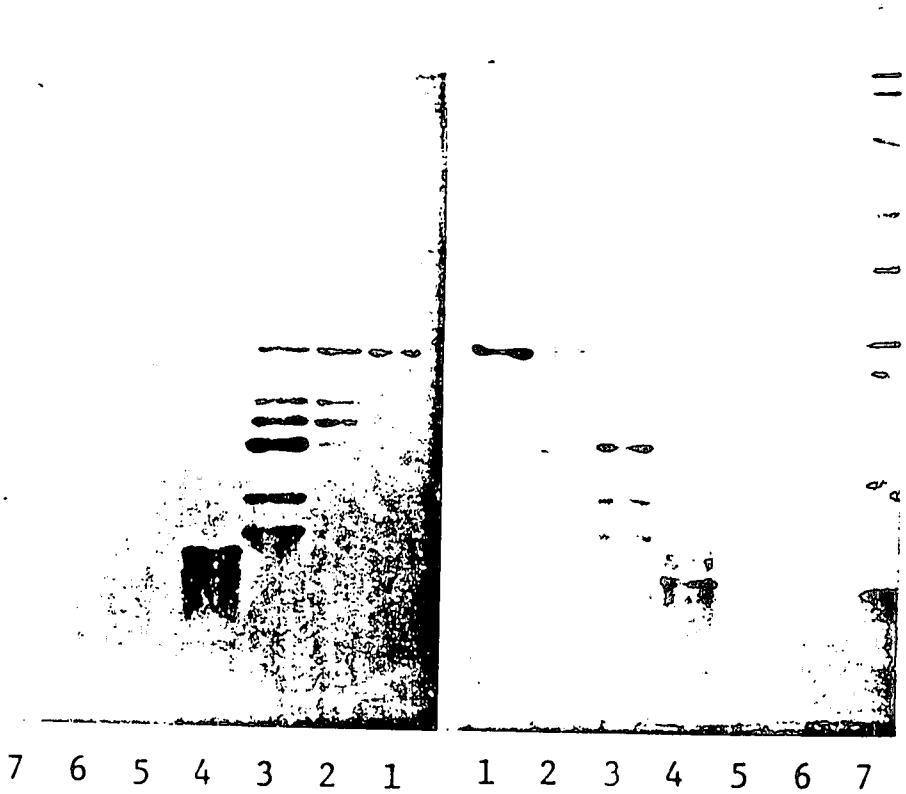


Fig. 3

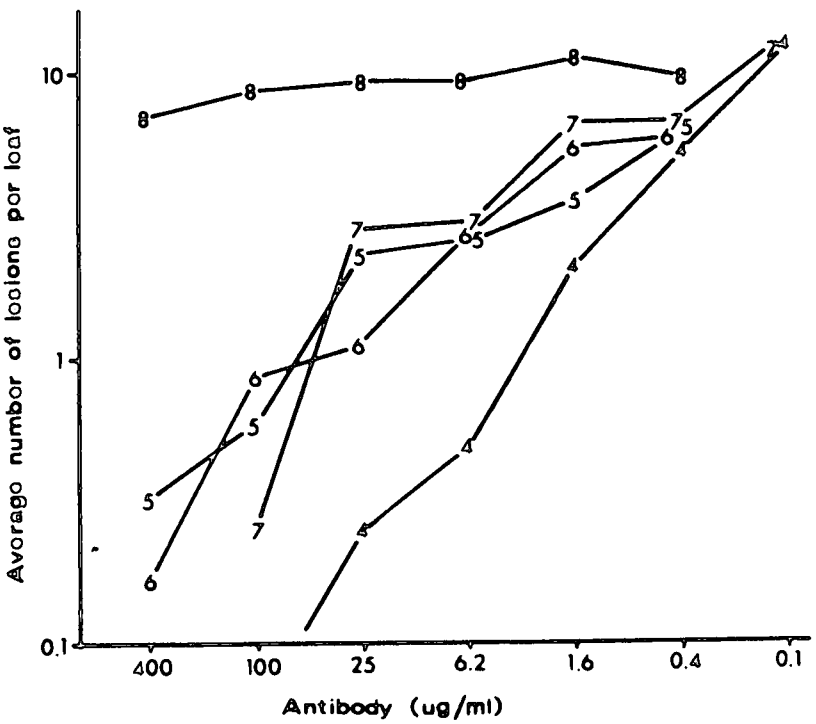
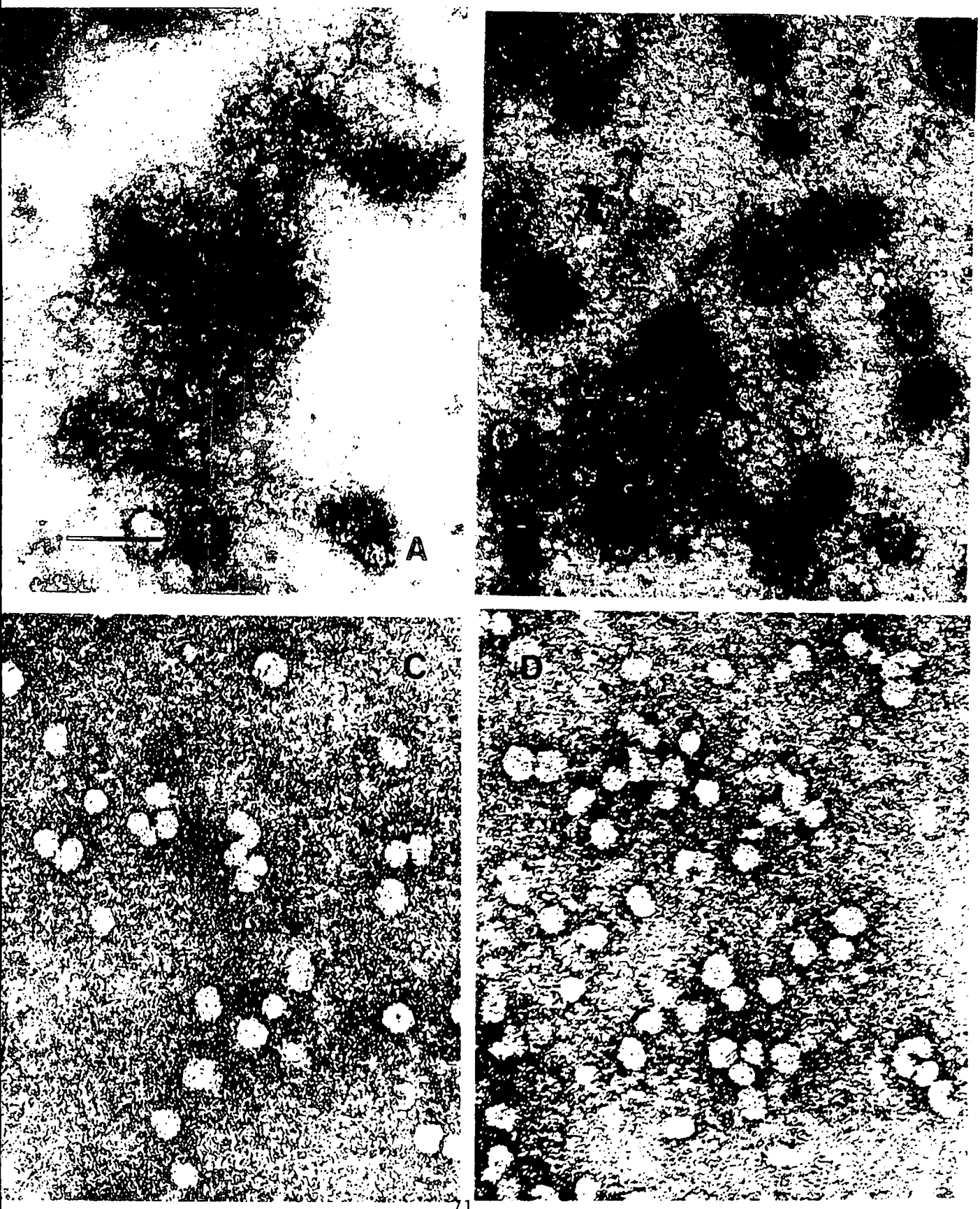

Average number of lesions per leaf
10
1
0.1
400
100
25
6.2
1.6
0.4
0.1
Antibody (ug/ml)

Fig. 4



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