Production of Monoclonal Antibodies to Viruses in the Potyvirus Group: Use in Radioimmunoassay

By E. K. HILL, J. H. HILL* AND D. P. DURAND
Department of Microbiology and *Department of Plant Pathology, Seed and Weed Science, Iowa State University, Ames, Iowa 50011, U.S.A.

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SUMMARY
Hybridomas were developed that secreted antibodies to soybean mosaic (SMV), lettuce mosaic (LMV) viruses, and to maize dwarf mosaic (MDMV) virus, strains Ap and B. All hybridomas produced antibodies specific to the homologous virus except for one to LMV, which produced antibodies that reacted at a low level with SMV and MDMV. Monoclonal antibodies against SMV were used in a double-antibody sandwich radioimmunoassay. The assay using only one type of monoclonal antibody lacked sensitivity because, presumably, limited epitopes were available. Results obtained using two monoclonal antibodies that bound to different epitopes were comparable to those using polyclonal antibodies. A competitive radioimmunoassay, using a single monoclonal antibody, was developed to detect successfully SMV, LMV and MDMV.

INTRODUCTION
Immunosorbent assays are widely used for the detection of plant viruses (Clark, 1981). Antisera utilized in these assays are often raised in rabbits and can frequently react with antigens from healthy plant tissue. Furthermore, it may be difficult to obtain antisera to viruses that are laborious to purify in sufficient quantities for production of polyclonal antisera.

We have recently developed a solid-phase radioimmunoassay (SPRIA) for the detection of soybean mosaic virus (SMV), which uses immunopurified polyclonal immunoglobulin G (IgG) produced in rabbits (Bryant et al., 1983). The objectives of the research we report were to develop hybridoma cell lines to selected members of the potyvirus group and compare polyclonal with monoclonal antibodies for detection of SMV using SPRIA. Adequate assay sensitivity required the use of two hybridomas specific to apparently different epitopes on the virus particle. Therefore, we developed a competitive radioimmunoassay (RIA) that is dependent upon a single monoclonal antibody and obviates the requirement for immunologically distinct hybridomas in SPRIA. The assay could detect SMV, lettuce mosaic virus (LMV) and maize dwarf mosaic virus (MDMV) at a concentration of 10 to 50 ng of purified virus per ml. Although monoclonal antibodies have been widely applied to the study of animal viruses (Koprowski et al., 1977; Flamand et al., 1980; Yelton & Scharff, 1981), they have received limited attention for the study of plant viruses (Al Moudallal et al., 1982; Briand et al., 1982; Dietzgen & Sander, 1982; Halk et al., 1982a, b; Diaco et al., 1983). A preliminary report of this work has appeared (Hill et al., 1983).

METHODS
Viruses. SMV (Ia 75-16-1) was purified from infected Glycine max (L.) Merr. cv. Williams as described previously (Hill & Benner, 1980). MDMV, strains Ap (Berger, 1980; Berger et al., 1983) and B (ATCC-PV53) were purified from Sorghum sudanese Piper (Stapf), cv. Trudan 5 (Northrup King Corp., Minneapolis, Mn., U.S.A.) and Zea mays L. cv. Golden Bantam, respectively, by a modification of the procedure of Langenberg (1973). Lettuce mosaic virus (LMV) (ATCC-PV63) was purified according to Ghabrial & Shepherd (1980). Virus concentrations were estimated spectrophotometrically by using A°620 = 2.4 (Purcifull, 1966). Production of monoclonal antibodies. BALB/c mice (Jackson Laboratories, Bar Harbor, Me. U.S.A.) were
Incubated for 1 h at 37 °C. Plates were washed twice with PBS-Tween, and 50 μl of alkaline phosphatase-labelled (1 mg/ml), was added. After incubation for 30 to 60 rain, reactions were determined visually or spectrophotometrically.

Zymed Laboratories\] was added. Positive wells turned blue green within 15 min; negative wells and controls remained colourless.

50 μl of peroxidase-labelled, affinity-purified goat anti-rabbit immunoglobulin (Zymed Laboratories) was added. Antigen. Control wells received normal rabbit serum. After 2 h incubation at 37 °C and washing with PBS-Tween, wells were rinsed and 50 μl of ABTS substrate solution [1 mM-2,2'-azino-di-3-(ethylbenzthiazoline sulphonic acid) in 0.1 M-citrate buffer, pH 4.2, containing 1 μl/ml 30% H2O2, Zymed Laboratories] was added. Positive wells turned blue-green within 15 min; negative wells and controls remained colourless.

Purification and tritium labelling of IgG. Monoclonal antibodies were purified by affinity chromatography of ascites fluid by using Protein A-Sepharose CL-4B (Pharmacia). IgG was bound using 0.1 M-Na2 HPO4 pH 8.0 and 100 μl foetal bovine serum, tool. wt. 1540 (PEG, American Type Culture Collection) in serum-free DMEM over a period of 30 s. After the cells had been gently agitated for 2 min, the PEG solution was diluted with 1 ml DMEM added during a 30 s period. After a second 2 min incubation, 10 ml of DMEM was added during 1 min with gentle agitation, and the cell suspension was incubated for 5 min. Cells, collected by centrifugation at 250 g for 10 min, were resuspended, at a density of 2.5 × 106 myeloma cells/ml, in a medium consisting of equal volumes of CM and DMEM (DMEM-CM) to which sufficient 100× hypoxanthine–aminopterin–thymidine (Dutchland Laboratories, Denver, Pa., U.S.A.) was added to achieve a final concentration of 1× (HAT medium).

The cell suspension was plated into 96-well (0.2 ml/well) tissue culture plates (CoStar) with a wide-mouth pipette. Plates were incubated at 37 °C in a humidified atmosphere enriched to 5 to 7% CO2 (the medium used would also support growth without CO2 enrichment). When the medium turned a yellow–orange colour (3 to 4 days), cells were fed by removing 0.1 ml of culture medium from each well and replacing it with HAT medium. One week after fusion, cells were slowly weaned by replacement of the HAT medium with HT medium (HT = HAT with no aminopterin; Dutchland Laboratories). Testing of culture supernatants by enzyme-linked immunosorbent assay (ELISA) (see below) was initiated 2 or 3 days after HT medium was added to the wells and when hybrid cells had grown sufficiently to cover one-fourth of the well. Specific antibody-producing hybridomas were cloned by limited dilution (Galfre & Milstein, 1981) into DMEM–CM and fed with DMEM. Positive cell lines were transferred to 25 cm2 CoStar tissue-culture flasks, fed with DMEM, and used for propagation in vitro of monoclonal antibody, or were frozen in Nunc cryogenic vials (Vanguard International, Neptune, N.J., U.S.A.) at a cell density of 1 × 108 to 2 × 108 cells/ml in foetal bovine serum containing 10% dimethyl sulphoxide at -70 °C and then stored in liquid nitrogen. Ascites fluid was obtained by intraperitoneal injection of BALB/c mice, primed 1 to 4 weeks earlier by intraperitoneal injection of 0.5 ml Pristane (2, 6,10,14-tetramethylpentadecane), with 0.5 × 107 to 1.0 × 107 hybridoma cells in 0.2 ml DMEM. When the abdomen was distended, the ascites fluid was collected by insertion of an 18-gauge needle into the peritoneal cavity; the fluid was centrifuged at 1000 g to remove cells.

ELISA. The indirect ELISA used for screening cell lines for specific monoclonal antibodies was similar to that described by Voller et al. (1979). Virus antigen (10 μg/ml) in 100 μl of carbonate coating buffer (0.05 M-NaHCO3, -Na2CO3, pH 9.6) was added to wells of microtitre plates (Immunon I) (Dynatech Laboratories, Inc., Alexandria, Va., U.S.A.), which were incubated for 1 h at 37 °C. Virus solution was removed and unbound protein-binding sites were blocked by adding to the wells 300 μl 0.01 M-sodium phosphate, 0.15 M-NaCl, pH 7.2 (PBS) containing 1% (w/v) bovine serum albumin (PBS-BSA) and incubating for 15 min at 37 °C. Plates were washed once with PBS containing 0.05% Tween 20 (PBS–Tween), and 50 to 100 μl of test sample was added to each well and incubated for 1 h at 37 °C. Plates were washed twice with PBS–TWEEN, and 50 μl of alkaline phosphatase-labelled anti-mouse IgG (Sigma, cat. no. A 1902) diluted 1:1500 in PBS–BSA was added. After incubation of plates for 1 h at 37 °C and four washes with PBS–TWEEN, 50 μl of p-nitrophenyl phosphate in 10% (v/v) diethanolamine, pH 9.8 (1 mg/ml), was added. After incubation for 30 to 60 min, reactions were determined visually or spectrophotometrically at 405 nm.

Determination of immunoglobulin subclass. A modification of the indirect ELISA was used to determine the IgG subclass of the monoclonal antibodies. A subclass-specific rabbit anti-mouse immunoglobulin (50 μl) (Zymed Laboratories, Burlingame, Ca., U.S.A.) was added to wells containing monoclonal antibody bound to virus antigen. Control wells received normal rabbit serum. After 2 h incubation at 37 °C and washing with PBS–TWEEN, 50 μl of peroxidase-labelled, affinity-purified goat anti-rabbit immunoglobulin (Zymed Laboratories) was added to each well. After another 2 h incubation at 37 °C, wells were rinsed and 50 μl of ABTS substrate solution [1 mM-2,2'-azino-di-3-(ethylbenzthiazoline sulphonic acid) in 0.1 M-citrate buffer, pH 4.2, containing 1 μl/ml 30% H2O2, Zymed Laboratories] was added. Positive wells turned blue-green within 15 min; negative wells and controls remained colourless.
eluted with 0.5% acetic acid in saline, pH 3.0. Concentrations of IgG were determined by assuming A_{280} = 1.4.

Samples were adjusted to pH 7.0 to 8.0 with 1 M NaOH, diluted 1:1 with glycerol, and stored at -20 °C until used.

Purified IgG was labelled with tritium by the sodium borohydride exchange reaction described previously (Bryant et al., 1983). Specific activities ranged between 3 and 18 μCi/mg protein.

Preparation of polyclonal IgG and SPRIA. Immunopurified rabbit polyclonal IgG (RP) was prepared as described previously (McLaughlin et al., 1980) and labelled with tritium. SPRIA, utilizing polyclonal and monoclonal antibodies, used procedures developed in this laboratory (Bryant et al., 1983). PBS-BSA was used as a blocking agent instead of PBS-Tween containing ovalbumin. Optimal antibody coating and ³H-labelled IgG concentrations used in the assay were obtained by calculation of binding ratios (Hill et al., 1981).

Competitive RIA. Polystyrene 'frosted' beads (3.4 mm diam.; Precision Plastic Ball Co., Chicago, Ill., U.S.A.) were coated with purified virus antigen at a concentration of 1 to 10 μg per bead in carbonate-coating buffer by gentle rotation for 20 h at room temperature. The coating solution was aspirated, and beads were washed three times (3 to 5 min) with PBS-Tween. Beads were incubated an additional h with PBS-BSA to block unbound protein-binding sites and washed again with PBS-Tween.

To assay virus, test samples were diluted in either 0.05 M-sodium borate, pH 7.2 (BB), containing 0.5% sodium metabisulfite, (NaPO₃)₁₃, for purified virus or BB for soybean seed extracts containing SMV prepared as described by Bryant et al. (1983). One ml of virus sample was added to 12 × 75 mm plastic tubes (Falcon) containing 0.2 ml of tritium-labelled antibody diluted in PBS-BSA. Plastic tubes had been pre-coated 1 to 2 h with PBS-BSA and rinsed three times with PBS-Tween. After incubation at room temperature with gentle agitation for 1 h, one antigen-coated bead was added to each tube, and incubation continued for an additional 1 h. The assay was terminated by aspiration of the sample, and the bead was washed as before. Beads were transferred individually into 5 ml of Riafluor scintillation cocktail (New England Nuclear) for measurement of bound ³H-labelled IgG. Satisfactory assays required prior determination of optimal concentrations of immobilized virus antigen and tritium-labelled IgG in the presence and absence of sample virus (Hill et al., 1981). Assays for SMV, LMV, MDMV-Ap and MDMV-B used the monoclonal antibodies S1, L5, MA1 and MB4, respectively.

RESULTS AND DISCUSSION

Preparation and characterization of monoclonal antibodies

Mouse antisera generally had a titre of 1:256 when tested against an antigen concentration of 1 mg purified antigen/ml, demonstrating that the mice responded reasonably well to virus antigen. ELISA titres of ascitic fluid from different cell lines varied from 1:10² to 1:10⁶. All antibodies (Table 1) contained kappa light chains and were members of the IgG2a, IgG2b or IgG3 subclasses.

Most of the monoclonal antibodies were specific for the homologous antigen. The L5

<table>
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<th>Clone designation</th>
<th>Immunizing virus</th>
<th>IgG subclass</th>
<th>ELISA titre to Immunizing virus</th>
<th>ELISA titre to heterologous virus</th>
<th>Protein A-purified IgG (mg) bound to 0.5 ml Protein A-Sepharose</th>
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<tr>
<td>S1</td>
<td>SMV</td>
<td>IgG2aK</td>
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<td>†</td>
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</tr>
<tr>
<td>S2</td>
<td>SMV</td>
<td>IgG2aK</td>
<td>1:10⁵</td>
<td>-</td>
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</tr>
<tr>
<td>L5</td>
<td>LMV</td>
<td>IgG2bK</td>
<td>1:10⁶</td>
<td>1:10²‡</td>
<td>1.7</td>
</tr>
<tr>
<td>MA1</td>
<td>MDMV-Ap</td>
<td>IgG3K</td>
<td>1:10⁴</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td>MB3</td>
<td>MDMV-B</td>
<td>IgG3K</td>
<td>1:10⁴</td>
<td>-</td>
<td>§</td>
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<tr>
<td>MB4</td>
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<td>IgG2bK</td>
<td>1:10⁵</td>
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<tr>
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<tr>
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<td>IgG2aK</td>
<td>1:10⁵</td>
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</table>

* ELISA titre was determined by using serial dilutions of clarified ascitic fluid in an indirect ELISA using 5 μg/ml virus antigen. All antibodies were also tested against purified tobacco mosaic and cowpea mosaic viruses, and no reaction was observed.
† No binding above background.
‡ Cross-reactions of L5 with SMV, MDMV-Ap and MDMV-B were equivalent.
§ Not determined.
monoclonal antibody, however, did cross-react with SMV, MDMV-Ap and MDMV-B (Table 1). ELISA titres of L5 were considerably lower with the heterologous antigens than with LMV, suggesting that the other three viruses have an epitope similar, but not identical, to the site on LMV. Although cross-reactivity is not unexpected among viruses of the same group, this is, to our knowledge, the first report of an apparently similar epitope on LMV, SMV and MDMV.

Experiments were performed to measure the binding affinity of Protein A-Sepharose for various monoclonal antibodies. Measured amounts of Protein A-purified antibody were added to 0.5 ml of Protein A-Sepharose until its capacity was exceeded. Results showed that binding affinity of Protein A–Sepharose for various monoclonal antibodies. Measured amounts of Protein A-purified antibody were added to 0.5 ml of Protein A–Sepharose until its capacity was exceeded. Results showed that binding affinity of Protein A, even for antibodies of the same subclass, varied by more than twofold (Table 1). These differences probably are caused by variations in the amino acid sequence of the Fc region of the molecule and reveal the individual nature of monoclonal antibodies.

**Utilization of monoclonal antibodies in double-sandwich SPRIA**

Initial experiments in which the same monoclonal antibody was used for coating the solid-phase polystyrene beads and for tritium labelling revealed that SPRIA using monoclonal antibodies had very poor sensitivity when compared with SPRIA using RP antibodies (Fig. 1). Later experiments revealed, however, that tritium-labelled S1 antibody (Table 1) bound to SMV better than labelled RP (Fig. 2). Experiments using a mixed double-sandwich SPRIA, which

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**Fig. 1.** Relationship between ct/min bound in solid-phase radioimmunoassay and concentration of purified soybean mosaic virus diluted in 0.05 M-sodium borate (pH 7.2) containing 0.5% (NaPO₃)₃. △, Coating rabbit polyclonal IgG (10 µg/bead) used with tritium-labelled rabbit polyclonal IgG (1 µg/bead). □, S1 coating monoclonal IgG (10 µg/bead) used with tritium-labelled S1 monoclonal IgG (1 µg/bead).

**Fig. 2.** Binding of tritium-labelled S1 monoclonal IgG (□) and tritium-labelled rabbit polyclonal IgG (△) to soybean mosaic virus immobilized on polystyrene beads. Virus was bound to polystyrene beads overnight in carbonate coating buffer. Tritium-labelled IgG (1 µg/bead) was added to beads and then incubated for 1 h.
incorporated S1 as coating antibody and RP as labelled antibody, or conversely, RP as coating antibody and S1 as labelled antibody, showed that S1 antibody was an efficient coating antibody for tritium-labelled RP, but that RP provided inadequate sensitivity when used as a coating antibody for labelled S1 (Fig. 3). These results suggested that when RP was used as the coating antibody the reduced binding of labelled S1 antibody was caused by binding of RP to epitopes specific to S1 antibody.

It seemed plausible that this problem could be circumvented by using a monoclonal antibody whose affinity for epitopes was different from that of S1. Monoclonal antibodies L5 and S2 have been shown to bind to SMV (Table 1). Experiments utilizing L5 or S1 as coating antibodies and S1 as the labelled antibody showed poor response to virus antigen. When S2 was used as coating antibody and S1 as labelled antibody, detection sensitivity, using previously described criteria (Bryant et al., 1983), was 25 ng/ml (Fig. 4). The poor response obtained in experiments incorporating L5 antibody may be due to the apparent low avidity of S5 for SMV. Comparison of experiments using S1 as coating and labelled antibody with those using S2 and S1 as coating and labelled antibody, respectively, indicates S2 binds to a different epitope on SMV than does S1. This was further suggested by a competitive radioimmunoassay. When labelled S1 was
added to antigen-coated beads previously exposed to either unlabelled S1 or S2, unlabelled S2 did not block binding of labelled S1 but unlabelled S1 reduced binding of labelled S1 fivefold.

Antigenic differences, detectable by ELISA, have been reported among SMV strains (Chen et al., 1982). Tests with SPRIA using RP showed cross-reactivity when isolates from strains G-1 to G-7 (Cho & Goodman, 1979) were tested (Bryant et al., 1983). When SMV-specific monoclonal antibodies S1 and S2 were tested against isolates G-1 to G-5, no differences were detected (data not shown). Although S1 and S2 distinguish between apparently different epitopes on SMV, it appeared that these epitopes are common to all SMV strains tested in this study.

Our results demonstrate that an effective SPRIA can be developed for detection of plant viruses using monoclonal antibodies. The direct double-sandwich assay requires IgG specific to apparently different epitopes on the virus particle and possesses sensitivity equivalent to that reported for SPRIA using RP IgG.

Utilization of monoclonal antibodies in competitive RIA

Experiments showed that a single monoclonal antibody could be used in a competitive RIA for detection of SMV, LMV and MDMV. The binding curve for detection of MDMV-Ap shows that the number of counts per minute was inversely proportional to the amount of virus in the sample (Fig. 5). Similar curves were obtained for MDMV-B, SMV and LMV (data not shown). Sensitivity of the assay, from 10 to 50 ng/ml of virus antigen, was similar to that of SPRIA using monoclonal and polyclonal antibodies (Byrant et al., 1983). The presence of soybean seed extract reduced the number of counts per minute bound by 40 to 45% when monoclonal antibody S1 was used in the assay, but sensitivity and quantitative relationships were retained (data not shown). Competitive assays have not generally been reliable diagnostic tests because of inhibition
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caused by plant sap when polyclonal antisera are used. The use of monoclonal antibodies may obviate some of these difficulties.

The large quantity of specific antibody that can be produced from a hybridoma is potentially attractive for preparing antibodies to plant viruses that are difficult to purify in sufficient quantity for production of polyclonal antisera. These results suggest that the use of monoclonal antibodies for plant virus detection in diagnostic and epidemiological studies is a feasible alternative to the use of polyclonal antisera.

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