Techniques

Detection of Homologous and Heterologous Barley Yellow Dwarf Virus Isolates with Monoclonal Antibodies in Serologically Specific Electron Microscopy

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ABSTRACT

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Monoclonal antibodies (M-Abs) produced against: a barley yellow dwarf virus (BYDV) isolate transmitted specifically by the aphid vector *Macrosiphum* (=*Sitobion*) *avenae* (MAV); an isolate transmitted specifically by *Rhopalosiphum padi* (RPV); or an isolate transmitted nonspecifically by both aphid vectors (PAV), were able to detect efficiently all BYDV isolates tested in serologically specific electron microscopy (SSEM). They did not, however, detect the unrelated soybean mosaic or cowpea mosaic viruses in SSEM. The procedure was highly sensitive, detecting as little as 7.5 pg of virus. Also, SSEM performed on a mixture of BYDV and the morphologically distinct soybean mosaic virus detected only BYDV particles. These M-Abs are specific for common, rather than group-specific, determinants because they are capable of binding to isolates from serological groups of BYDV previously regarded as serologically distinct. In SSEM, they should be useful in screening a broad range of BYDV isolates, even in mixed infections with other viruses.

Additional key words: luteovirus, serology.

Barley yellow dwarf virus (BYDV) is an economically significant plant virus that causes disease world wide in barley, wheat, oats, and many perennial grasses (6,17). Transmission of the virus is aphid-dependent. Several distinct types have been identified, based upon specificity of aphid transmission (12,16). They are exemplified by five isolates referred to as: MAV, which is specifically transmissible by *Macrosiphum* (=*Sitobion*) *avenae* Fabricius; RPV, which is specifically transmissible by *Rhopalosiphum padi* L.; PAV, which is nonspecifically transmissible by both vectors; RMV, which is specifically transmissible by *R. maidis* Fitch; and SGV, which is specifically transmissible by *Schizaphis graminis* Rondani (12,16,17). Replication of the virus is phloem-restricted (11,15), and BYDV occurs in very low concentrations in infected plants. Detection and differentiation of BYDV isolates has always been a difficult task. Diagnosis based solely on symptoms is unreliable because other agents can induce similar host reactions (1,7,19). Additionally, BYDV-infected plants can be symptomless (10) or have symptoms masked by summer temperatures (7). Differentiation schemes based on aphid transmission tests are time-consuming and laborious (13) because they require repeated acquisition and transmission tests (16). Serological testing by ELISA, although faster and less labor-intensive, has required testing of each sample with multiple antisera to ascertain the presence, or absence, of each BYDV isolate (13,18). These procedures are also subject to the limitations of available polyclonal antisera, and have limited sensitivity.

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Serologically specific electron microscopy (SSEM) (3), combining the specificity of serology with the sensitivity of electron microscopy, has been applied to the detection of BYDV by MAV-specific polyclonal antiserum (14). The procedure was inefficient for detecting the heterologous RPV and RMV isolates of BYDV. Some monoclonal antibodies (M-Abs) produced against BYDV have been shown to cross-react with heterologous BYDV isolates (4,5). This report describes efficient detection of a PAV-like isolate ("P-PAV" [8]), and the PAV ("RC-PAV"), MAV, and RPV isolates of Rochow (16,17) with single monoclonal antibody (M-Ab) preparations in SSEM. This provides a distinct advantage over the polyclonal-antibody-based SSEM described by Paliwal (14) and is the first report of successful use of M-Ab in SSEM of plant viruses. A preliminary report has appeared (4).

MATERIALS AND METHODS

Virus and monoclonal antibodies. The RPV, MAV, P-PAV, and RC-PAV isolates of BYDV used were purified at Purdue University as previously described (8) and shipped frozen to Iowa State University. The unrelated soybean mosaic virus (SMV), strain Ia 75-16-1, and cowpea mosaic virus (CPMV), strain Sb, were purified as described by Hill and Benner (9) and Van Kammen (22), respectively.

The monoclonal antibody preparations P-PAV 1D7, MAV 4F7, and RPV 3F10 (4,5), were independently produced against the P-PAV, MAV, and RPV isolates of BYDV, respectively. Four-week-old Balb/C mice (Charles River Breeding Laboratories, Wilmington, MA), two for each isolate, were injected intraperitoneally with 0.3–0.4 ml of a suspension containing 50 μg of purified virus (with an extinction coefficient of 5.6 $[mg/ml])^{-1} \cdot cm^{-1}$ at 260 nm) emulsified in Freund's complete adjuvant, followed by an additional 25 μg of virus in 0.1 M sodium phosphate, pH 6.0, 4–6 wk later. Four days after hyperimmunization, the mice were exsanguinated, and the spleen cells were fused by using a modification of previously described methods (21). When hybrid cells had grown sufficiently to cover one-fourth of the well, specific-antibody-producing hybridomas were cloned three times by limiting dilution. Cloned cell lines were then transferred to 25 cm^3 CoStar tissue-culture flasks and were used for in vitro propagation of monoclonal antibody. Once established, cell lines were frozen at −70 C in Nunc cryogenic vials (Vanguard International, Neptune, NJ) at a cell density of $1–2 \times 10^6$ cells per milliliter in fetal bovine serum containing 10% dimethylsulfoxide and stored in liquid nitrogen. Ascitic fluid was obtained by intraperitoneally injecting Balb/C mice, primed 3–4 wk earlier by intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane), with $0.5–1.0 \times 10^7$ hybridoma cells in 0.2 ml of Dulbecco's modified Eagle's medium. When the abdomen was distended, the ascitic fluid was collected by insertion of an 18-gauge needle into the peritoneal cavity. The ascitic fluid was clarified by centrifugation at 1,000 *g* to remove cells, and the fluid was stored frozen at −20 C or with 0.1% NaN_3 at 4 C.

Monoclonal antibodies were purified by affinity chromatography of ascitic fluid with protein-A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). Immunoglobulin G (IgG) was bound by using 0.1 M sodium phospate, pH 8.0, and eluted with 5.0% acetic acid in saline, pH 3.0. Concentrations of IgG were determined by an extinction coefficient of 1.4 $(mg/ml)^{-1} \cdot cm^{-1}$ at 280 nm. Samples were adjusted to pH 7.0–8.0 with NaOH and were stored as described for ascitic fluid.

Electron microscopy. Nitrocellulose-coated carbon-stabilized 200-mesh copper grids were floated for 30 min at room temperature (21 C) on 20-μl drops of M-Ab diluted in 0.05 M tris, pH 7.2, containing 0.02% polyvinylpyrrolidone 40 (PVP-40; Sigma Chemical Co., St. Louis, MO) and 0.15 M NaCl (TPN buffer). Dilutions of M-Ab from 10 to 0.08 μg/ml were compared for BYDV adsorption efficiency. For the three M-Ab preparations used, dilutions of 1–3 μg/ml of M-Ab adsorbed the homologous isolates most efficiently (*unpublished*). Therefore, 1 μg/ml solutions of M-Ab were used to coat all grids. The M-Ab-coated grids were washed, dropwise, with 2 ml of TPN buffer, then drained on Whatman no. 1 filter paper, and floated on 10-μl drops of sample diluted in 0.01 M sodium phosphate buffer, pH 7.0 (phosphate buffer). The grids were then incubated for 30 min at room temperature in closed dishes containing moistened filter paper.

Samples consisted of purified virus, purified virus diluted in healthy plant extract, or healthy plant extract alone. The healthy plant extract was prepared by first thoroughly grinding liquid-nitrogen-frozen *Avena sativa* L. 'Clintland 64' oat tissue with a prechilled mortar and pestle, followed by further grinding (1:6, w/v) in 0.1 M sodium phosphate, pH 6.0. Immediately before use, samples were subjected to centrifugation for 5 min at 8,740 *g* in a Beckman Microfuge B (Beckman Instruments, Palo Alto, CA). This removed particulate matter from the samples and resulted in much "cleaner" grids. The grids were washed, as above, with 2 ml of phosphate buffer and 2 ml of deionized H_2O, and were stained, dropwise, with 1 ml of 1% aqueous uranyl acetate. After draining and air drying, the grids were examined in an Hitachi HU-11C electron microscope at 75 kV. Mean virus particle counts were obtained by a procedure similar to that of Paliwal (14). Virus particles were counted in five 70 × 80 mm average fields of view, at a magnification of ×38,000, in each of three randomly selected grid squares. Two identically treated grids were counted for each sample; the preparation and viewing of samples were always performed at random. Mean virus particle counts (MVPC) refer to the average number of particles from 30 viewing areas.

Detection efficiency of heterologous BYDV isolates. To determine the detection efficiency of the homologous and heterologous BYDV isolates, purified virus was diluted to 50 ng/ml in healthy plant extract, and the MVPCs were determined. Analyses of variance, and contrasts (20), comparing the sample totals and means, were performed on the data to compare adsorption of the different BYDV isolates by each M-Ab preparation. The sensitivities of the different M-Ab preparations for the homologous isolates were determined by performing SSEM on serial 10-fold dilutions containing from 750 ng to 0.75 pg of purified virus per milliliter of plant extract. The limit of sensitivity of each M-Ab preparation was taken as the lowest dilution showing an MVPC of five or greater. This dilution would have a reasonable probability of showing at least one virus particle per average field of view at ×38,000.

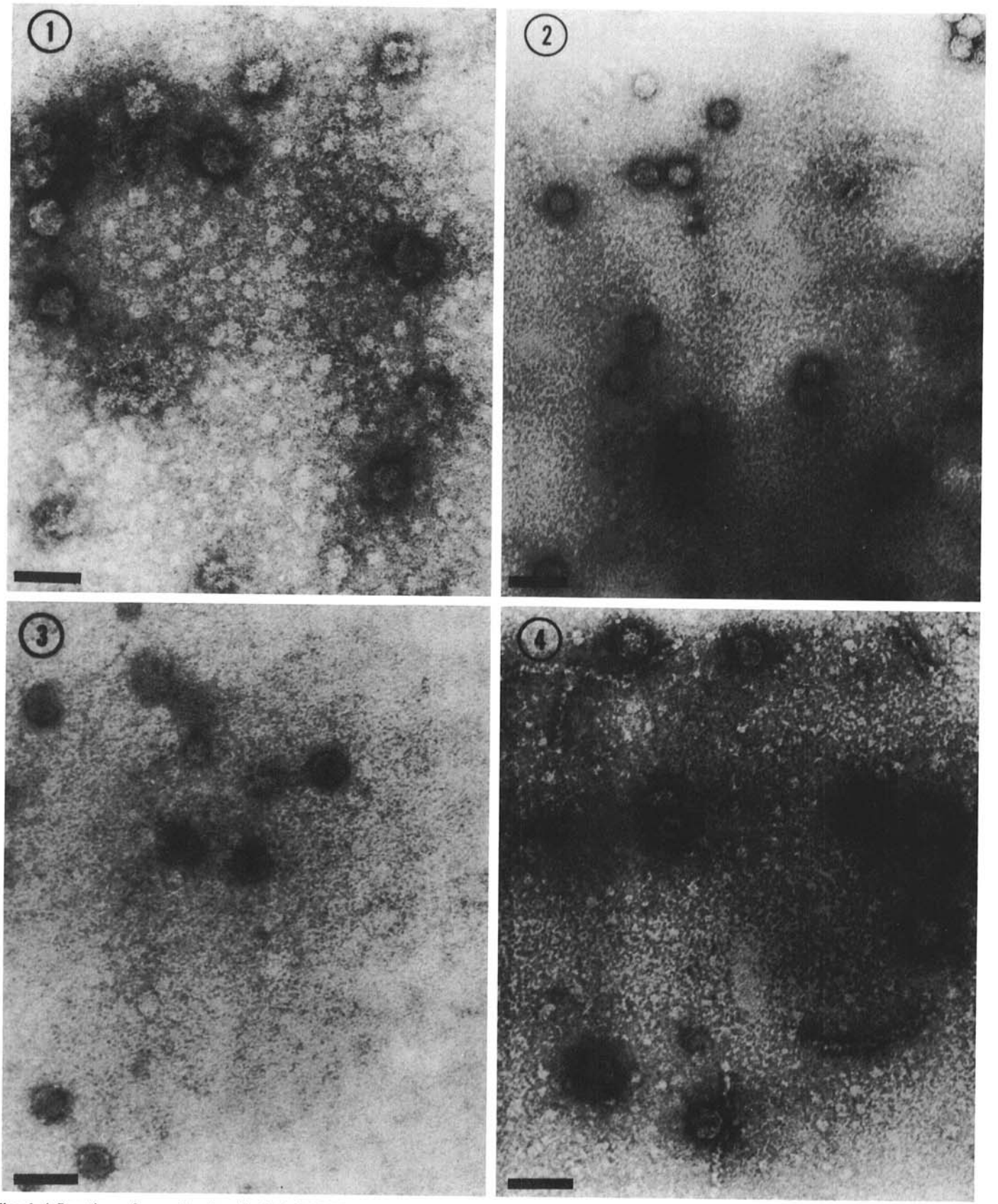
To assess the capability of the M-Ab based SSEM procedure to detect each of the isolates in infected tissues, Clintland 64 oat plants containing single BYDV isolates were extracted and examined by SSEM, using M-Ab P-PAV 1D7 as the immunosorbent. As controls, grids coated with pre-immune mouse sera were used as the immunosorbent, and also the unrelated cowpea mosaic and soybean mosaic viruses were reacted with M-Ab-coated grids. In another experiment to assess the specificity of the SSEM procedure, purified SMV and BYDV were mixed and examined by SSEM.

RESULTS

Assay conditions. In preliminary experiments, some grids incubated for 2 hr or longer at 37 C oxidized in the presence of TPN buffer and became contaminated with copper salts. To circumvent this problem, shorter incubation times were used for adsorbing M-Abs to the grids. This did not adversely affect the binding characteristics of the serologically specific grids; enough M-Ab was bound to the grids during a 30-min incubation period to efficiently adsorb BYDV isolates and preclude nonspecific adsorption of unrelated virus. The grids were not adversely affected by the presence of the phosphate buffer in which the virus was diluted, even after 3 hr at 37 C. Incubation of BYDV with serologically specific grids at 37 C slightly increased ($P < 0.05$) the number of virions entrapped over that obtained at room temperature (e.g., 424 and 392 virions, respectively, from 75 ng/ml solutions of P-PAV with M-Ab P-PAV 1D7 as the immunosorbent), but some particles appeared degraded. With longer incubation times, the numbers of particles entrapped were not significantly increased. However, the adsorption of nonspecific debris increased with longer incubations.

A standard procedure was adopted in which test samples were incubated with M-Ab-coated grids for 30 min at room temperature. To aid further in preventing nonspecific adsorption of particulate matter, the samples were subjected to low-speed (8,740 *g*) centrifugation for 5 min immediately prior to incubation with the M-Ab coated grids. This treatment was extremely effective in removing cellular debris that interfered with particle counting.

Detection of homologous and heterologous BYDV isolates. To compare the detection efficiency of homologous and heterologous BYDV isolates by the M-Ab preparations, purified virus



Figs. 1–4. Reactions of monoclonal antibody P-PAV 1D7 to the P-PAV isolate of barley yellow dwarf virus in serologically specific electron microscopy with 75 ng/ml solutions of: **1,** P-PAV; **2,** RC-PAV; **3,** MAV; and **4,** RPV. Bar = 50 nm.

preparations were diluted to 50 ng/ml with healthy plant extract and reacted with M-Ab-coated grids under the optimized conditions described above. The results (Table 1) show that each of the M-Abs could efficiently adsorb homologous and heterologous BYDV isolates. Figs. 1–4 show the reactions of M-Ab P-PAV 1D7 with 75 ng/ml solutions of the P-PAV, RC-PAV, MAV, and RPV isolates of BYDV. Statistical analyses of these results indicated that the BYDV isolates were not adsorbed equally by any of the M-Ab preparations (Table 2). However, these M-Ab preparations did not specifically adsorb the unrelated CPMV or SMV (Figs. 5–8). Also, control grids coated with pre-immune mouse serum did not readily adsorb BYDV particles; mean virus particle counts with such grids did not exceed two particles per viewing area. When BYDV was

TABLE 1. Mean virus particle counts in serologically specific electron microscopy (SSEM) and detection efficiency for barley yellow dwarf virus (BYDV) isolates with three monoclonal antibody preparations

BYDV isolate	Mean virus particle count[a] (percent detection efficiency)[b] obtained with monoclonal antibody preparation:		
	P-PAV-1D7	MAV 4F7	RPV 3F10
P-PAV	263.3 ± 10.0 (100)	132.2 ± 5.0 (82.3)	80.3 ± 2.6 (84.5)
RC-PAV	251.5 ± 11.2 (95.5)	127.2 ± 7.5 (79.2)	79.0 ± 2.7 (83.2)
MAV	245.8 ± 11.6 (93.3)	160.7 ± 10.9 (100)	89.8 ± 5.6 (94.5)
RPV	225.3 ± 9.9 (85.6)	112.5 ± 12.3 (90.0)	95.0 ± 5.4 (100)

[a] The mean virus particle counts were from five 70 × 80-mm viewing areas at an electron microscope magnification of ×38,000. Counts ± standard deviations taken from three randomly selected grid squares on two identically treated grids.

[b] Percent detection efficiency = (mean heterologous virus particle count)/(mean homologous virus particle count) × 100.

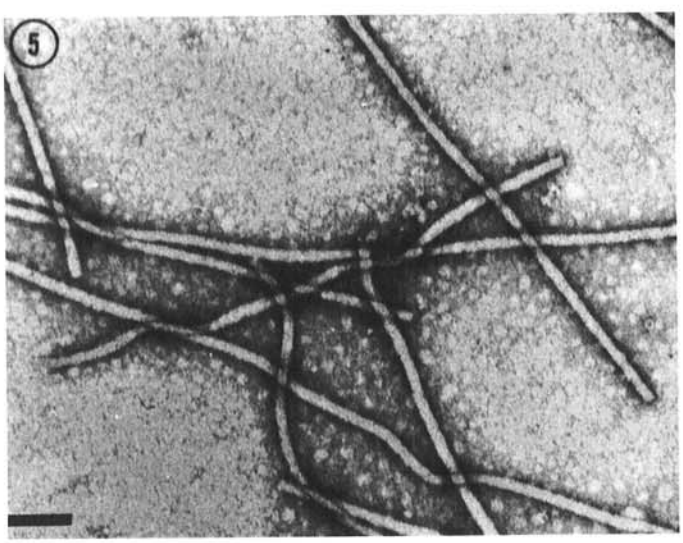
TABLE 2. Comparison between mean virus particle counts obtained with barley yellow dwarf virus (BYDV) isolates by using three monoclonal antibody preparations

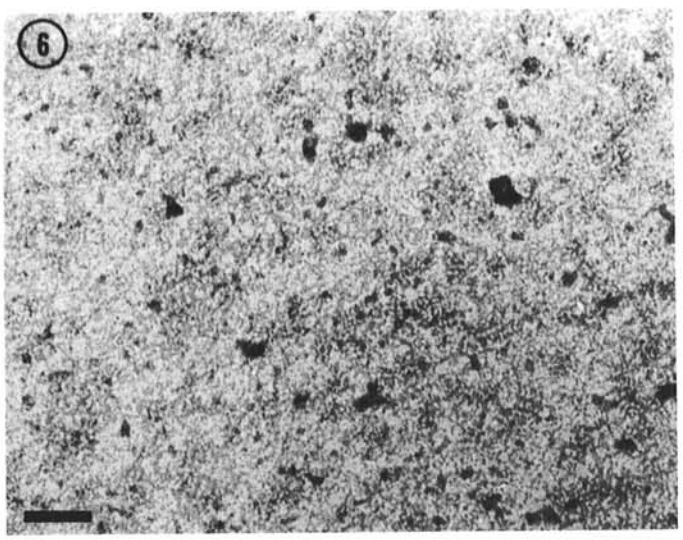
BYDV isolates compared[a]	Immunosorbent[b]	Probability[c]
PAV and MAV	P-PAV 1D7	$P > 0.100$
PAV and RPV	P-PAV 1D7	$P < 0.010$
MAV and RPV	P-PAV 1D7	$P < 0.050$
PAV and MAV	MAV 4F7	$P < 0.005$
PAV and RPV	MAV 4F7	$P < 0.010$
MAV and RPV	MAV 4F7	$P < 0.005$
PAV and MAV	RPV 3F10	$P < 0.005$
PAV and RPV	RPV 3F10	$P < 0.005$
MAV and RPV	RPV 3F10	$P < 0.050$

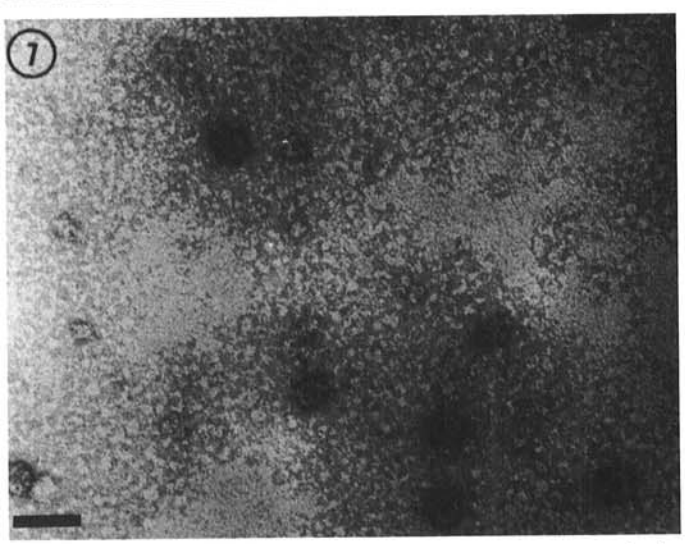
[a] Mean virus particle counts were compared by analysis of variance and contrasts (20).

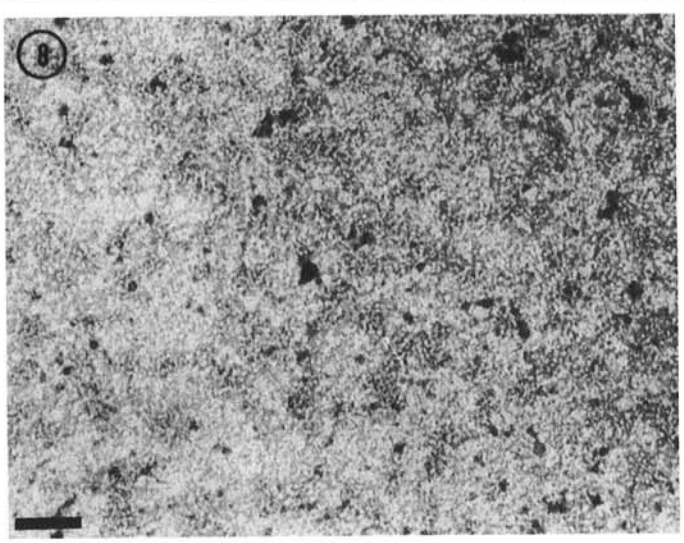
[b] Monoclonal antibody preparation used in SSEM procedure at 1 μg/ml.

[c] Probabilities <0.050 are significant, $P < 0.01$ are highly significant, with respect to the indicated pairs of compared isolates being adsorbed with different efficiencies.









Figs. 5–8. Negative stains of 150 ng/ml solutions of **5,** soybean mosaic virus and **7,** cowpea mosaic virus and reaction of grids treated with monoclonal antibody P-PAV 1D7 to the P-PAV isolate of barley yellow dwarf virus in serologically specific electron microscopy with these solutions (Figs. 6 and 8 for soybean mosaic virus and cowpea mosaic virus, repectively). Bar = 50 nm.

mixed with the morphologically distinct SMV and examined by SSEM on M-Ab P-PAV 1D7-coated grids, only BYDV particles were adsorbed to the grids (Figs. 9–10).

Sensitivity of SSEM. The sensitivity of each M-Ab preparation was determined for the homologous isolate by reacting various dilutions of purified virus, made in healthy plant extract, with M-Ab coated grids. The lowest virus concentrations yielding an MVPC of five or greater, were 0.75 ng/ml for M-Ab P-PAV 1D7 (MVPC = 5.3), 7.5 ng/ml for M-Ab MAV 4F7 (MVPC = 26.7), and 7.5 ng/ml for M-Ab RPV 3F10 (MVPC = 16.3), respectively, although in each case, virus particles could still be detected in further dilutions. The MVPC for each M-Ab preparation were linear in the range of 7.5–150 ng/ml (*unpublished*). As an indication of the sensitivity of the test with infected plants, extracts (1:6, w/v) of laboratory-inoculated Clintland 64 oat plants, containing each of the BYDV isolates, were examined by SSEM using M-Ab P-PAV 1D7. The MVPCs obtained for P-PAV, MAV, and RPV infected plants were 588, 446, and 297, respectively.

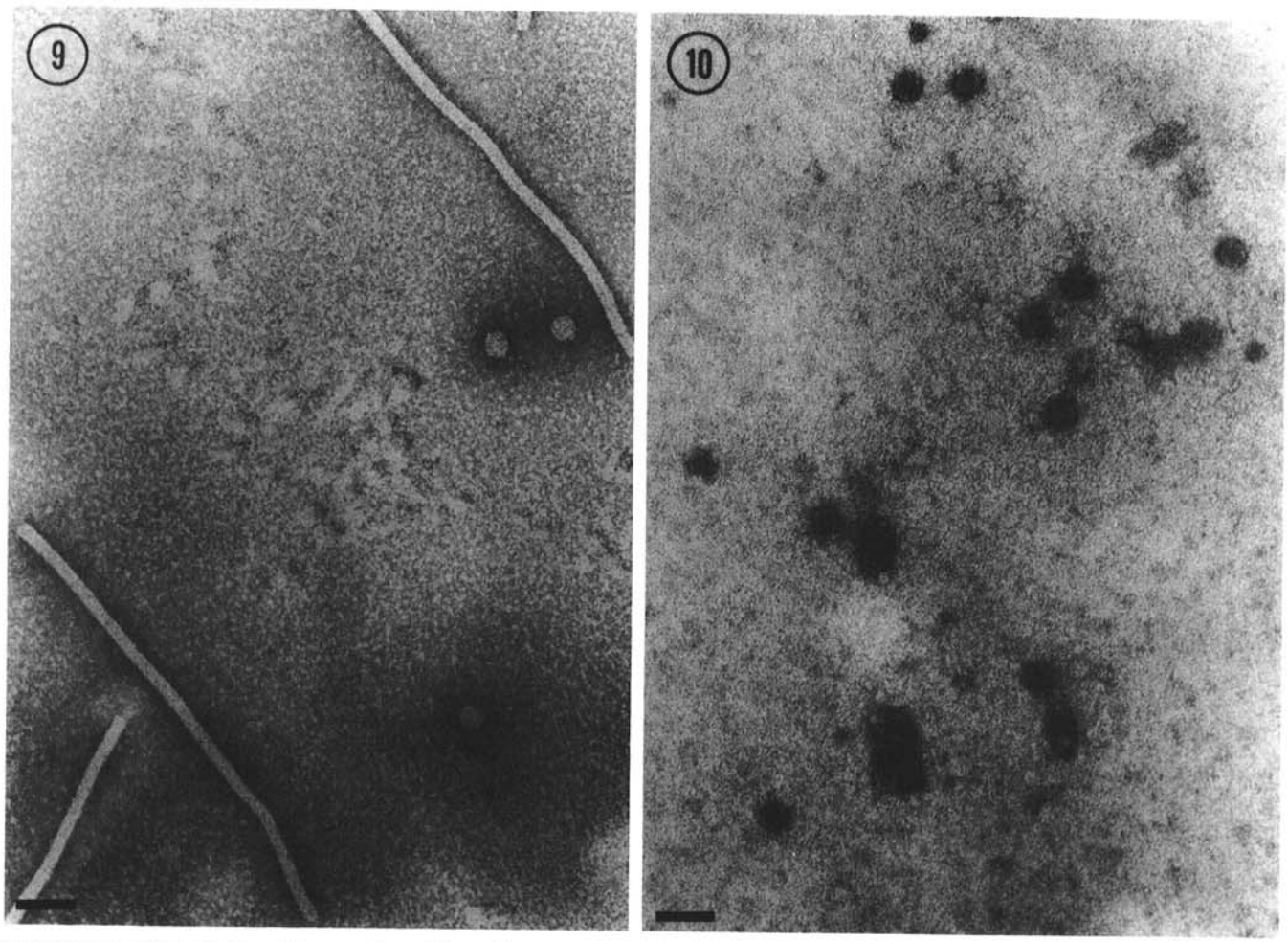
DISCUSSION

Only when M-Ab was used in the SSEM procedure were BYDV particles specifically adsorbed and detected by electron microscopy. Sodium chloride was omitted from buffers used for virus extraction and dilution to avoid aggregation and uneven distribution of virus particles (2). Paliwal (14) diluted BYDV-infected tissues in 0.05 M tris-HCl, pH 7.2, containing 0.02% PVP-40 and 0.9% NaCl, but, in our experiments, BYDV particles were degraded after prolonged incubation in this buffer. However, they were stable for long incubation periods when diluted in phosphate buffer, and they were dispersed evenly on the surface of the grids.

Centrifugation of the samples prior to incubation with antibody-coated grids, and extensive washing with buffer and deionized water, were helpful in removing contaminating particulates. These steps were necessary to easily distinguish the small isometric BYDV particles.

Optimal antibody concentrations and incubation conditions were required to achieve high sensitivity and specificity with the system. For the three M-Ab preparations described in this study, solutions containing purified M-Ab at approximately 1 μg/ml gave optimal adsorption of homologous virus. These concentrations efficiently bound heterologous BYDV isolates, but not the unrelated viruses that were tested. Specificity was established by exposing grids treated with M-Ab to preparations of SMV or CPMV and also to mixtures of BYDV with twice the concentration of SMV. In each case, only BYDV particles were specifically bound to the grids, suggesting adsorption of BYDV was not due simply to electrostatic attraction. Control grids coated with pre-immune mouse sera did not specifically bind BYDV or the other viruses.

Reactions in various serological test procedures, including gel diffusion and ELISA, have been used to separate BYDV isolates into two groups, each comprising isolates sharing group-specific epitopes. Thus, RPV and RMV isolates share epitopes that distinguish them from the interrelated PAV, MAV, and SGV isolates (17). However, in the SSEM procedure previously described (14), polyclonal antiserum to an MAV-like isolate, while reacting strongly with MAV-, PAV-, and SGV-like isolates, reacted weakly with RMV- and RPV-like isolates, suggesting an



Figs. 9–10. 9, Negative stain of a mixture containing 75 ng of the PAV-like (P-PAV) isolate of barley yellow dwarf virus and 150 ng of soybean mosaic virus per milliliter; and **10,** reaction of grids treated with monoclonal antibody P-PAV 1D7 to barley yellow dwarf virus in serologically specific electron microscopy with this solution. Bar = 50 nm.

epitope common to all five kinds of isolates. In this case, the reduced reactivity of the MAV- specific antiserum for RMV- and RPV-like isolates could indicate that the concentration of immunoglobulin molecules specific for common BYDV epitopes was low, and was overshadowed by that of immunoglobulin molecules specific for MAV, PAV, and SGV group-specific epitopes. The monoclonal antibodies we have produced react with isolates from both serological groups (i.e., PAV, MAV, and RPV) and therefore react with common rather than group-specific epitopes (4, 5, and *unpublished*). They should, therefore, be useful for screening a wide range of BYDV isolates and perhaps may also react with isolates of the SGV and RMV type.

With all three M-Abs, the MVPC was highest with homologous BYDV, but the MVPC obtained with heterologous BYDV isolates was at least 70% of that obtained with equivalent concentrations of homologous BYDV. Indeed, analysis of variance and contrasts (20) indicated no difference in the MVPCs obtained when M-Ab P-PAV 1D7 was used to detect the PAV or MAV isolates. But, differences in MVPC were obtained with this M-Ab when detection of the PAV and RPV isolates was compared and, similarly, with the MAV and RPV isolates. With M-Abs MAV 4F7 and RPV 3F10, differences were obtained in comparisons of MVPCs between all three of these isolates. In other experiments, however, there were no differences in comparisons of the MVPCs obtained with the three M-Abs and the P-PAV and RC-PAV isolates ($P > 0.100$).

M-Ab P-PAV 1D7 was the most sensitive of the M-Abs, detecting virus particles in solutions containing virus 0.75 ng/ml. M-Abs MAV 4F7 and RPV 3F10 detected virus particles in solutions containing 7.5 ng of virus per milliliter. Since the grids were exposed to 10 μl of sample, only 7.5 pg of virus were required for detection by P-PAV antibody 1D7 and 75 pg for the MAV 4F7 or RPV 3F10 antibodies. The differences in the MVPC obtained with the three M-Abs probably reflect differences in antibody affinities rather than in sensitivity or specificity. The antibody-coated grids were reacted with sample for only 30 min; under such conditions, antibodies with stronger affinities would adsorb more virus particles. The MVPC obtained from laboratory-inoculated Clintland 64 oat plants containing single BYDV isolates was high, suggesting sufficient sensitivity in the assay for detection of BYDV-infected plants. Because virus content varies with host, duration of infection, and other factors, assessment of SSEM sensitivity with plant extracts will require extensive investigations, but it seems likely to be similar to or exceed that of ELISA.

The data indicate that SSEM utilizing selected M-Abs is a sensitive and specific procedure to detect BYDV-infected plants containing virus isolates like PAV, MAV, or RPV and perhaps others. It should also be useful in detecting BYDV in mixed infections with other viruses. It should serve as a useful tool for epidemiological studies such as estimating the frequency of BYDV infection, especially in areas where more than one isolate of BYDV is endemic. Since the assay involves monoclonal rather than polyclonal antiserum, further production of BYDV-specific immunoglobulin is now greatly simplified.

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