Southern Bean Mosaic Virus Monoclonal Antibodies: Reactivity with Virus Strains and with the Virus Antigen in Different Conformations

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ABSTRACT

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Monoclonal antibodies B4 to B10 were produced against southern bean mosaic virus bean-type strain and C1 and C3 against the cowpea-type strain. They were tested against virus, swollen virus, and virus protein antigens from five bean strains and four cowpea strains in gel diffusion, immunoelectron microscopy, indirect enzyme-linked immunosorbent assay (ELISA), double antibody sandwich (DAS)-ELISA, antigen competition ELISA, and latex agglutination tests. B5, B6, and B10 were predominantly virus reactors with bean strains only. They precipitated virus, but not swollen virus and protein, in gel diffusion and immunoelectron microscopy; they reacted well with virus in indirect ELISA, but B6 and B10 had lower reactivities with protein and with swollen virus and protein, respectively. B4, B7, B8, B9, C1, and C3 were predominantly swollen virus and protein reactors with both bean and cowpea strains. They did not react with virus, swollen virus, or protein in gel diffusion or immunoelectron microscopy but reacted with swollen virus and protein and weakly with virus in indirect ELISA. B7 and C1 reacted strongly with virus in DAS-ELISA and B7 reacted with virus in latex agglutination tests. However, B7 and B9 were inhibited by swollen virus and protein but not by virus in competitive inhibition assays. The reaction of these antibodies with virus in indirect ELISA, latex agglutination tests, and DAS-ELISA was attributed to denaturation of virus on polystyrene plates and latex beads or by reaction with polyclonal antibodies in DAS-ELISA. The binding sites of B5, B6, and B10 on the virion of the bean-type strain were not blocked by the reaction of the virus with trinitrobenzenesulfonic acid.

Southern bean mosaic virus (SBMV) has a narrow host range confined almost exclusively to species of Leguminosae (14). Isolates that infect bean rarely infect cowpea and vice versa. The virus has been reported in the Americas, Africa, and southern Europe and some of these strains were available to us. Virus particles are stable over a wide pH range but become swollen in EDTA at pH 7 to 8 (10) and dissociate into separable RNA and protein components with further addition of NaCl to 1 M (16). The cowpea-type strain (SBMV-c) has been studied extensively by amino acid sequencing and X-ray diffraction (5,9).

The purpose of the studies reported here was to test the reactivity of monoclonal antibodies prepared against the bean-type strain (SBMV-b) and SBMV-c with native virus, swollen virus, and virus protein by several serological methods. Reactivity of four additional bean strains and three additional cowpea strains was also assessed. Preliminary reports of these studies have been made (17,18).

MATERIALS AND METHODS

Viruses and purification. The sources of cultures of SBMV-b, SBMV-c, the severe Mexican bean strain (SBMV-m), and the Ghana cowpea strain (SBMV-g) were given previously (19). The bean strains SBMV-br, SBMV-co, and SBMV-a were obtained from: F. P. Cupertino, Brazil, S. A. (3); F. Morales, Colombia S. A.; and J. P. Fulton, Arkansas (the A strain [20]), respectively. The cowpea strains, SBMV-n and SBMV-k, were obtained from G. Thottappilly, Nigeria, and C. W. Kuhn, Georgia (8), respectively. Strains SBMV-b, SBMV-m, SBMV-r, and SBMV-co were multiplied in *Phaseolus vulgaris* L. 'Bountiful' and SBMV-a in cultivar Pinto. Strains SBMV-c, SBMV-g, SBMV-n, and SBMV-k were multiplied in *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'Blackeye'. All viruses were purified by the pH 5.0 method (19) described previously except that a sucrose density gradient centrifugation and an extra cycle of differential centrifugation were used. The viruses were suspended in 0.01 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.02% sodium azide (PBS). All bean strains and the cowpea strains, SBMV-n and SBMV-k, were very soluble in this buffer (greater than 30 mg/ml) but the cowpea strains, SBMV-c and SBMV-g, could not be dissolved from high-speed pellets at concentrations greater than 8 mg/ml.

Antigen preparation. Five forms of virus antigen were used in serological tests: native virus (V), swollen virus (S), virus protein (P), SDS-denatured virus (D), and formic acid protein (F). The S antigen was prepared from V antigen by incubation in PBS, 15 mM EDTA, for 30 min at 0 C; the P antigen was prepared from the S antigen by adding NaCl to 1 M and leaving for 1 hr at 0 C. The V and S antigens (300 μg) were purified by sucrose density gradient centrifugation for 2 hr at 174,000 *g* in a 10–40% sucrose gradient in PBS for V or in PBS, 3 mM EDTA for S. The P antigen (300 μg) was laid over a 2-ml cushion of 10% sucrose in 0.5 M NaCl, topped with 0.5 M NaCl, and centrifuged in the same run. These antigens were used the day of their preparation. The D antigen was prepared from the S antigen by boiling for 2 min in 1% SDS. V, S, P, and D antigens were diluted to 20 μg/ml in PBS. The F antigen was prepared with virus in 66% formic acid at 37 C for 24 hr. After dialysis against two 1-L changes of distilled water, the F antigen was diluted to 20 μg/ml in distilled water. The F antigen was at pH 2.5.

The trinitrobenzene sulfonic acid(TNBS)-virus antigen was prepared in 3 ml of 0.04 M sodium phosphate buffer, pH 8.0, containing 8.8 mg of SBMV-b V or SBMV-c V and 2 mg of TNBS. The reaction was monitored at 345 nm for 16 hr in a Gilford model 250 recording spectrophotometer with a TNBS buffer solution used as a blank. $A_{345\ nm}$ increased for 8 hr then leveled off. The degree of trinitrophenylation was calculated by the method of Goldfarb (4,6) to be 0.88 and 0.84 moles of lysine reacted per mole of virus subunit of SBMV-b and SBMV-c, respectively.

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The TNBS-reacted SBMV-b and SBMV-c samples were chromatographed on a 2.5 × 20-cm column of Sephadex G-50 in the phosphate buffer. The recovered viruses had absorbances at 260 nm and 345 nm which indicated a reaction of 2.01 and 2.04 moles of lysine per virus protein subunit of SBMV-b and SBMV-c, respectively. The TNBS-virus particles appeared normal in electron micrographs and sedimented the same distance as native virus in sucrose density gradients. SBMV-c has two residues of lysine per subunit on the virion surface (5).

Immunization and cell fusion. Balb/c mice were immunized at weekly intervals with three to five intraperitoneal injections of 100 μg of purified virus in 0.5 ml of PBS or PBS Freund's complete adjuvant. Spleen cells were fused with NS-1 mouse myeloma cells at a ratio of 10:1 with 1 ml of 50% PEG-1500 (BDH Chemicals, Vancouver, BC, Canada). Fused cells were resuspended in Dulbecco's modified Eagles' minimum essential medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 20% fetal calf serum (FCS) (Flow Laboratories, McLean, VA) with 5×10^5 BALB/c feeder thymocytes per milliliter. Aliquots of cell suspension were seeded into 96-well culture plates and grown in a 37 C humidified incubator in a 10% CO_2:90% air atmosphere. Supernatant fluids from the culture wells were assayed for antibody activity against native virus, swollen virus, and viral coat protein by an indirect enzyme-linked immunosorbent assay (ELISA) after 1–2 wk. Selected antibody-secreting hybridomas were transferred to 24-well culture plates and retested. Positive cultures were cloned by the limiting dilution method. Cell lines were stored frozen in liquid nitrogen in medium containing 20% FCS and 10% dimethylsulfoxide. Hybridoma cells were propagated as ascitic tumors in mice previously injected with pristane (Sigma). Ascitic fluid was clarified by low-speed centrifugation and frozen at −20 C. Thawed ascitic fluid was reclarified and chromatographed on a Sephacryl S300 column (2.5 × 90 cm) equilibrated in 10 mM tris-Cl, 0.5 M NaCl, 0.02% NaN_3. Fractions containing antibody activity were precipitated with $(NH_4)_2SO_4$, dissolved in PBS containing 0.02% NaN_3, then stored at −20 C after the addition of 33% gylcerol. The Vancouver Research Station hybridoma designations are VRS-H-(011-017)-SBMV-b for B4, B5, B6, B7, B8, B9, and B10 antibodies and VRS-H-(019-020)-SBMV-c for C1 and C3 antibodies. Antibodies B5, B7, B8, B9, C1, and C3 were IgM whereas B6 and B10 were IgG immunoglobulin.

ELISA. Antibody-secreting hybidomas were detected by indirect ELISA. Purified antigens were diluted to a concentration of 20 μg/ml in PBS and 100-μl aliquots were placed in 96-well Linbro EIA plates and allowed to stand for 16–24 hr at 4 C. The wells were rinsed extensively with water and nonspecific protein-binding sites were quenched by adding 300 μl of ELISA buffer (PBS pH 7.5, 0.2% BSA, 0.02% NaN_3, 0.05% Tween-20) for at least 1 hr at room temperature. Purified antibodies were diluted in ELISA buffer to $A_{280\ nm} = 0.1$ and then ten-fold dilutions were made. Antigen-coated wells were incubated for 60 min with 100 μl of purified monoclonal antibody dilutions, washed with water, and subsequently incubated with 100 μl alkaline phosphatase-conjugated goat-anti-mouse Ig (200 ng/ml; Kirkegaard and Perry Lab. Inc., Gaithersburg, MD) in ELISA buffer for 90 min at room temperature. After a final rinsing, the wells were treated with 100 μl of *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) at a concentration of 0.5 mg/ml in 1.0 M diethanolamine, pH 9.8, for 90 min in a 37 C humidified incubator. The $A_{405\ nm}$ of each well was measured with a Titertek Multiskan MC plate reader connected to an Apple IIe microcomputer. IgG or IgM from ascitic fluid of mice injected with NS-1 were used as control tests and $A_{405\ nm}$ values from these controls were subtracted from appropriate test results. One antigen was tested on one plate with all antibodies. Each experiment consisted of eight plates for the four antigens of SBMV-b and SBMV-c and one plate of bovine serum albumin controls and this was done three times. Tests with the other bean and cowpea strains were done once. The data were fitted by computer to the equation

$$A = A_{max}\, X/(X + K_m)$$

in which A is the absorbance obtained with antibody dilution X, A_{max} is the maximum absorbance obtained, and K_m is the dilution at which one-half A_{max} is obtained. To distinguish between high titers obtained with low or high values of A_{max}, the reactivity parameter used was A_{max} divided by K_m. The results of one experiment is presented in Table 1, but the relative reactivities of each antibody with V, P, and S antigens in the three experiments were similar.

For double antibody sandwich (DAS)-ELISA, the protocol was the same except the plates were coated with 10 μg of anti-SBMV-b rabbit globulin per milliliter at pH 9.6 and the antigens were in ELISA buffer. The experiment was done twice with similar results.

Competitive antigen inhibition assays (15) were used to study the binding of various antigens by protein-reactive antibodies B7 and B9 and the reaction of V-precipitating antibodies B5, B6, and B10 to V antigen of the various strains of SBMV. Briefly, samples of viral antigen were serially diluted into 120-μl volumes of ELISA buffer containing monoclonal antibody. A concentration of antibody used was chosen to give 75–80% saturation by the indirect ELISA. After a 60-min incubation of monoclonal antibody plus free antigen, 100-μl aliquots were transferred to antigen-coated EIA plates and the level of unbound antibody was determined by using the indirect ELISA protocol. For antibodies B5, B6, and B10, V antigen of SBMV-b was used as the immobilized antigen, and for the B7 and B9 antibodies, the S antigen was the solid-phase antigen.

Other serological methods. Gel diffusion serological tests were done by agar gel double diffusion on collodion-coated glass microscope slides. Antibody titers of ascitic fluids were determined by pipetting 40 μl of twofold serial dilutions in PBS into wells adjacent to wells containing 40 μl of SBMV-b (or other strain) at 200 μg/ml. Agar gels for V antigen tests contained 0.15 M NaCl; those for S antigen tests contained 20 mM EDTA, pH 7.5; and those for P antigen tests contained 20 mM EDTA and 0.4 M NaCl, pH 7.5 (12). After some tests, the agar gels were washed for 4 days in PBS. The gels were stained for 1 hr in 0.03% amido black 10B in a 5% acetic acid, 5% glycerol solution. Destaining to a desired transparency was done in 1% glycerol, 0.85% NaCl, 0.02% sodium azide. The gel was floated off the glass slide, placed on filter paper, and dried.

For immunoelectron microscopy, the ascitic fluids were prepared in a series of twofold dilutions from 1/32 to 1/4,096 in 0.06 M sodium phosphate buffer, pH 7.0, and 16 μl drops of each dilution were placed on dental wax. Antigen, 8 μl of SBMV-b at 10 μg/ml, was mixed with the ascitic fluid dilutions and left at room temperature for 4 hr. A carbon-coated, filmed grid was touched to the antiserum virus mixture; stained with 2% ammonium molybdate, pH 7.0; and examined in an electron microscope.

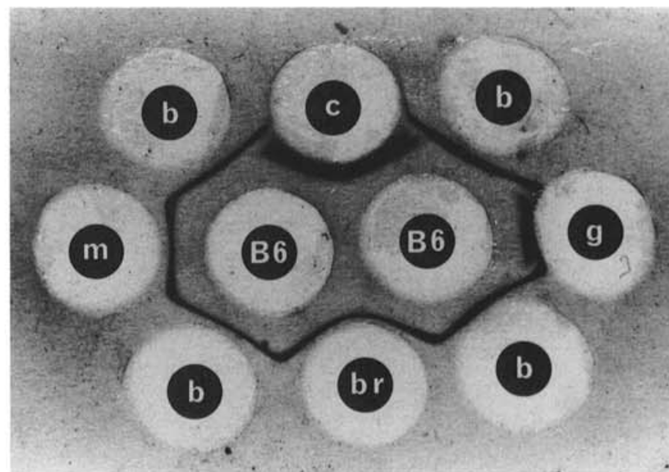

Fig. 1. Gel diffusion serological test with B6 monoclonal antibodies against southern bean mosaic virus (SBMV) bean-type strain in the central wells and virus antigens: b, bean strain; m, severe Mexican strain; br, Brazil strain; g, Ghana strain; and c cowpea strain in the outer wells. The gel was washed, fixed, and stained.

TABLE 1. Reaction of monoclonal antibodies with virus, swollen virus, virus protein, and SDS-denatured virus from the bean-type strain and cowpea-type strains of southern bean mosaic virus

Antigen	Values of A_{max}/K_m[a]								
	B5	B6	B10	B4	B7	B8	B9	C1	C3
Bean-type strain									
Virus	960	24,400	1,800	70	750	630	30	670	690
Swollen virus	980	20,800	800	530	3,600	3,500	270	1,100	2,700
Protein	890	3,000	6	700	7,300	6,400	250	1,300	4,850
SDS-protein	18	5	2	160	1,100	780	80	340	580
Cowpea-type strain									
Virus	4	210	3	160	650	570	40	500	830
Swollen virus	10	70	1	240	920	720	70	1,000	650
Protein	6	15	2	310	4,800	6,000	260	1,200	4,800
SDS-protein	10	5	1	240	730	980	210	320	530

[a] A_{max}/K_m is the maximum absorbance attained in indirect ELISA divided by the antibody dilution that produced one half of the maximum absorbance.

TABLE 2. Latex agglutination test with southern bean mosaic virus bean-type strain and monoclonal antibodies B7 and B10

Antibody	Dilution of antibody	μg/ml of virus[a]					
		0	5	10	20	40	80
B7	1/10	–[b]	–	–	++	++	++
	1/25	–	–	++	++	++	++
	1/50	–	–	+	++	++	++
	1/100	–	–	–	+	+	+
	1/200	–	–	–	–	–	–
B10	1/10	–	–	–	+	+++	+++
	1/25	–	–	+	++	+++	+++
	1/50	–	–	+	++	+++	+++
	1/100	–	–	+	++	+++	+++
	1/200	–	–	+	++	+++	+++

[a] The latex particles were treated with southern bean mosaic virus at indicated concentrations. The particles were washed twice in PBS and added to the dilution of monoclonal antibody indicated.
[b] A visual rating of agglutination obtained: –, none; +, little; ++, moderate; +++, heavy.

TABLE 3. Tests of monoclonal antibodies B7 and C1 in reactions with virion and protein antigens of southern bean mosaic virus, b strain, in double antibody sandwich ELISA

Monoclonal antibody	Antigens	Absorbance at 405 nm with an antibody dilution of:			
		1/10	1/100	1/1,00	1/10,000
B7	Virus	1.437	0.400	0.091	0.013
	Protein	0.744	0.362	0.068	0.010
C1	Virus	0.732	0.170	0.027	0.005
	Protein	0.562	0.233	0.036	0.072
Control[a]	Virus	0.054	0.004	0.003	0.008
	Protein	0.046	0.004	0.007	0.006

[a] IgM purified from ascitic fluid of mice injected with NS-1 myeloma cells and used in dilutions of 0.1, 0.01, 0.001, and 0.0001 $A_{280\ nm}$.

The latex agglutination test was performed according to the technique of Bercks and Querfurth (1); the SBMV-b V antigen was adsorbed to the latex, washed, and dilutions of ascitic fluid were added.

RESULTS

Gel diffusion and immunoelectron microscopy. Gel diffusion tests of all monoclonal antibodies were done with SBMV-b V, S, and P antigens. All tests with S and P were negative. With the V antigen, dilution end points of crude ascitic fluids were 1/512 for B5; 1/1,024 for B6; and 1/512 for B10. The other monoclonal antibodies were negative. B5 and B10 were negative with the V antigens of all cowpea strains, but an unusual precipitin pattern was observed when B6 was tested with bean and cowpea strain V antigens (Fig. 1). V antigens of the bean strains formed precipitin bands not touching the antigen well and these bands coalesced with bands in adjacent wells containing bean strain V antigens. Precipitates close to the antigen wells of the cowpea strains did not coalesce with the precipitin bands of the bean strain Precipitin patterns in tests with purified immunoglobulins were similar.

TABLE 4. Reaction of monoclonal antibodies B5, B6, and B10 with TNBS-reacted virions in indirect ELISA

Monclonal antibody	Antigen	Absorbance at 405 nm with an antibody dilution of:		
		1/10	1/100	1/1,000
B5	Virus	0.860	0.453	0.095
	TNBS-virus	0.738	0.479	0.085
B6	Virus	0.675	0.692	0.357
	TNBS-virus	0.603	0.577	0.249
B10	Virus	1.281	0.645	0.148
	TNBS-virus	1.041	0.691	0.121
IgG[a] Control	Virus	0.062	0.040	0.043
	TNVS-virus	0.098	0.127	0.078
IgM[a] Control	Virus	0.203	0.091	0.051
	TNBS-virus	0.269	0.107	0.083

[a] IgG or IgM purified from ascitic fluid from mice injected with NS-1 myeloma cells and used in dilutions of 0.1, 0.01, and 0.001 $A_{280\ nm}$. Appropriate control $A_{405\ nm}$ values have been subtracted from the values shown for B5, B6, and B10.

Only B5, B6, and B10 decorated the SBMV-b V antigen in immunoelectron microscopy. The SBMV-c V antigen was not reactive with these clones in IEM.

Indirect ELISA. The reaction of monoclonal antibodies with the V, S, P, and D in indirect ELISA is presented in Table 1 as the maximum absorbance attained in each test divided by the antibody dilution that produced one-half the maximum absorbance (A_{max}/K_m). Each of the V-precipitating antibodies, B5, B6, and B10 had different reactivities. The A_{max}/K_m values for B5 and for V, S, and P were almost equal. The B6 value with P was much lower than with V or S. The B10 value with V was much greater than with S and the value with P was negligible.

The remaining antibodies could be divided into two groups based on their A_{max}/K_m values with V, S, and P. B7, B8, and C3 had the greatest values with P, S was 50%, and V was 10% of this value. B4, B9, and C1 had similar values with S and P. The A_{max}/K_m values for V relative to the value for S or P were 10% for B4 and B9 and 50% for C1.

The reaction of B5, B6, and B10 with SBMV-c antigens was minimal. Relative to the SBMV-b antigens, A_{max}/K_m values were lower for SBMV-c S antigen with B4, B7, B8, B9, and C3 and lower for SBMV-c P antigen with B4 and B7. The SBMV-c virion has 60 more Ca^{++} binding sites than SBMV-b (9) and SBMV-c virions may be more stable.

The reactivities of the antibodies with the SDS-denatured virus (D) (Table 1) were similar to those with formic acid protein. B5, B6, and B10 did not react with D. The A_{max}/K_m values for the other antibodies with D were 12 to 31% of the values for the maximum-reacting antigen form. B4 and B9 appeared to react better with SBMV-c D than with SBMV-b D.

Indirect ELISA results with the monoclonal antibodies and V, S, and P antigens of the bean strains (SBMV-m, SBMV-br, SBMV-co, and SBMV-a) and with the cowpea strains (SBMV-n, SBMV-k, and SBMV-g) were very similar to those (Table 1) with SBMV-b and SBMV-c, respectively.

Latex agglutination tests. A moderate amount of agglutination was observed at higher V antigen coating levels and at higher concentrations of B7 (Table 2). Agglutination with B10 was greater at higher antigen coating levels and the dilution end point of B10 was greater than that of B7.

Reaction in double antibody sandwich ELISA. The reaction of B7 and C1 with SBMV-b V and P antigens was assessed by DAS-ELISA (Table 3). Both antibodies reacted with the V antigen to a greater extent than with the P antigen at the 1/10 dilution. However the reactivities of V and P were similar at the 1/100 dilution. The relative values for V and P were similar: 51 and 65 for B7; and 32 and 36 for C1.

Effect of TNBS reaction on virus reactivity with monoclonal antibodies B5, B6, and B10. Reaction of SBMV-b virions with TNBS had little effect on the reaction with virion-precipitating antibodies B5, B6, and B10 (Table 4). The $A_{405 nm}$ values at the 1/10 dilution are approximately 20% greater for the virus than for the TNBS-reacted virus. At the 1/100 dilution with B5 and B10, the $A_{405 nm}$ values are 5% greater for the TNBS-reacted virus. Apparently the reaction with TNBS did not alter the configuration of the antigenic sites of these antibodies appreciably.

Antigen inhibition tests. The "apparent" inhibition constant (K_i, the concentration in micrograms per milliliter of competing antigen required to produce a 50% inhibition of the observed antibody binding) was determined. The V antigen at concentrations up to 1,000 μg/ml did not inhibit binding of B7 or B9 to the immobilized S antigen. The K_i values for B7 were 398 for the S antigen and 58 for the P antigen and for B9 were 59 for the S antigen and 30 for the P antigen.

B5, B6, and B10 were not inhibited by V antigen of cowpea strains SBMV-c, SBMV-g, SBMV-n, or SBMV-k. The K_i values for V antigens of bean strains SBMV-b, SBMV-br, SBMV-co, and SBMV-a were 3.5, 3.5, 3.7, and 3.3 for B5; 8.1, 8.5, 8.6, and 4.8 for B6; and 0.65, 0.48, 0.70, and 0.59 for B10.

DISCUSSION

The weak reactions observed with the V antigen in indirect ELISA with B4, B7, B8, B9, C1, and C3 and in the latex agglutination test with B7 is in direct contrast to the lack of reaction in the liquid-phase tests: gel diffusion, immunoelectron microscopy, and antigen inhibition ELISA. The V antigen was probably partly denatured by the adsorptive forces holding them to the polystyrene plate or to the latex (polystyrene) beads. Similarly, the reaction of the plating antibody with the V antigen in DAS-ELISA may also distort virus particles. Therefore B4, B7, B8, B9, C1, and C3 probably have antigenic sites on the virus protein which are not available for reaction on the native virus particle. These antibodies were probably induced by dissociation of the virus particle after injection in the mouse. The B4 and B7 appear to react slightly better with the P antigen of SBMV-b than with that of SBMV-c. However, reactivities of B8, B9, C1, and C3 with the P antigens of SBMV-b and SBMV-c are similar.

Cross reactions between distantly related viruses are detected better with polyclonal protein-reactive antibodies than with virion-reactive antibodies (11,17). Chen et al (2) did not detect cross reactions between carnation ringspot and red clover necrotic mosaic viruses by gel diffusion or immunoelectron microscopy. Hiruki et al (7) detected weak cross reactions between these viruses in indirect ELISA and we detected stronger cross reactions with virus proteins of dianthoviruses or of sobemoviruses in indirect ELISA (13,17). Therefore, protein-reactive monoclonal antibodies could be of considerable value in determining or verifying weak polyclonal cross reactions. Protein-reactive monoclonal antibodies would also be useful in detection of protein subunits in sectioned material in electron microscopy. The use of both virus- and protein-reactive monoclonal antibodies may increase the sensitivity of virus infection indexing procedures if the viral protein is present in infected plant sap.

The reaction of B6 with the V antigen of SBMV-c in gel diffusion (Fig. 1) appears to be an artifact because no inhibition of B6 was obtained with this antigen in antigen inhibition tests.

Assuming that TNBS reacted with all of the sites on all of the virions, our serological tests indicate that B5, B6, and B10 do not react at these sites. The simple TNBS reaction with other plant viruses may be useful in determining whether monoclonal antibodies have distinctly different reactive sites.

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