Techniques

Properties and Potential Applications of Syngeneic Monoclonal Anti-Idiotypic Antibodies: Use as a Reference Standard in Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Syngeneic anti-idiotypic monoclonal antibodies were generated to an anti-soybean mosaic virus (SMV) monoclonal antibody. One of these antibodies (1a) was used in place of SMV to capture rabbit polyclonal anti-SMV antibodies in an enzyme-linked immunosorbent assay (ELISA). This demonstrated that an anti-idiotypic monoclonal antibody can be used as a standard in ELISA for detection of exotic pathogens when conditions preclude introduction of the pathogen into the environment.

Additional keyword: serology.

Serological assays have been particularly useful for identifying and detecting viruses with high sensitivity and accuracy. Although antivirus antibodies can be easily distributed, quarantines restrict distribution of exotic viruses, which are necessary positive controls. However, in lieu of the virus antigen, an antigen-mimicking or anti-idiotypic antibody would overcome this difficulty. We demonstrated that anti-idiotypic antibodies can be used as positive controls in immunological assays. We explain this concept from an immunological point of view and describe the production and characterization of these antibodies. In this study, soybean mosaic virus (SMV) was used as a model system.

An antigen may possess one or more distinct antigenic sites known as epitopes (17). In response to an antigen, an animal may produce antibodies that consist of a pair of high- and low-molecular weight peptide chains (Fig. 1). Each chain consists of segments of amino acids that are conserved (constant regions) and others that vary considerably (hypervariable regions) from one antibody to another (35). The antigen-combining sites (paratopes) of an antibody are located within the hypervariable region of an antibody (17).

An animal can also produce antibodies to antigenic sites on other antibodies. Antibodies that recognize the hypervariable region of an antibody are known as anti-idiotypic antibodies. An idiotype is composed of one or more idiotopes, which specifically define antigenic sites within an antibody's hypervariable region (17,24,32). In a similar fashion, antibodies that are reactive with constant-region domains of other antibodies (i.e., domains associated with an antibody's isotype or allotype) are termed anti-isotype or anti-allotype antibodies, respectively (20,35). These concepts are illustrated diagrammatically in Figure 1.

In 1974, Jerne proposed the immune network theory to describe the immune response in an antigenically stimulated animal (17). Within the animal, the paratope of an antibody (Ab1) can recognize the original immunizing antigen as well as an idiotope on other antibodies (Ab2 β) (23). Ab2 β molecules are also referred to as antigen-mimicking antibodies (42), internal-image antibodies (17,18,21,26), or homobodies (3,4,21) (Fig. 2). The idiotopes present on Ab1 may also be recognized by the paratopes of other antibody (Ab2) molecules. These Ab2 molecules are referred to as anti-idiotope or anti-idiotypic antibodies (23,33). Occasionally, in the same animal species, an idiotope on Ab1 may also be expressed on other antibodies whose antigenic specificity differs from Ab1. These idiotopes are termed cross-reactive idiotopes (11,25,28,40). Antibodies to the same antigen collected from

different animal species may also share a common cross-reactive idiotope (29,37,41).

These concepts were used to develop an antibody-based positive control for an enzyme-linked immunosorbent assay (ELISA), which, like most plant virus detection systems, uses rabbit polyclonal antibodies to detect virus antigen.

MATERIALS AND METHODS

Immunological reagents and viruses. The BALB/c mouse anti-SMV IgG2a kappa light-chain monoclonal antibody S1 (S1-MAb) has been previously described (15). Protein-A isolation and biotinylation of S1-MAb were performed as described (6). IgG2a kappa light-chain MAb produced against maize dwarf mosaic virus (MAG II-MAb) has been described (19), and IgG2a kappa light-chain antibodies (NDV4F11-MAb, NDV3H4-MAb) against Newcastle disease virus were produced in our laboratory. All were affinity-isolated on protein-A columns. Polyclonal antibodies to SMV, tobacco ringspot virus (TRSV), and the RPV isolate of barley yellow dwarf virus (BYDV) raised in New Zealand white rabbits were isolated by protein-A chromatography (6). Biotinylation of SMV polyclonal antibodies (SMV Ab) was performed as described (6). Concentration of purified antibodies was determined spectrophotometrically by using $A^{0.1\%}_{280nm} = 1.4$. The Ia 75-16-1 isolate of SMV was purified as described elsewhere (12).

Production and cloning of antibody-secreting hybridomas. Antibody-secreting hybridomas and mouse ascitic fluid were generated, as described by Van Deusen and Whetstone (39), by using SP2/0-Ag14 (SP2/0) myeloma cells and BALB/c mice as a source for primed spleen cells and ascitic fluid. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% serum (2:1, horse/calf) and were cloned according to Mernaugh et al (27).

Glutaraldehyde fixation of S1 hybridoma cells. Although hybridomas generally secrete antibodies and do not present them on the cell surface, glutaraldehyde fixation of a hybridoma should arrest antibodies in, on, or near the cell surface. Such antibodies will then be available to stimulate an immune response. Therefore, S1 hybridoma cells were fixed with glutaraldehyde by a modification of the procedure proposed by Guillet et al (10). Twenty to 30 ml of S1 hybridoma cells (approximately 3–5 $\times$ 10^7 cells), which grew in DMEM, were rinsed several times with 0.1 M potassium phosphate containing 0.15 M NaCl, pH 7.1 (PBS), and fixed for 15 min with 25 ml of PBS containing 1% glutaraldehyde (Polysciences, Warrington, PA; 1909). The cells were centrifuged at 250 *g* and resuspended in 50 ml of 0.2 M glycine for 2 hr to block remaining aldehyde groups. The cells were centrifuged again, resuspended in PBS containing 0.02% sodium azide, and refrigerated until used. Cells stored in PBS that contained azide were rinsed briefly with azide-free PBS before injection into mice.

Immunization of mice for generation of anti-idiotope antibodies to S1-MAb. Five BALB/c mice were immunized with S1 hybridoma cells by the following schedule. On day 0, the mice were injected intraperitoneally with 2.5 $\times$ 10^6 glutaraldehyde-fixed S1 hybridoma cells in PBS emulsified (1:1, v/v) with Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO; F-5881). They were then injected intraperitoneally with 5 $\times$ 10^6 fixed cells in PBS on day 19 (10). On day 33, an intraperitoneal injection of 55 μg of protein-A purified S1-MAb diluted in PBS was given. The last two injections of S1-MAb were given on days 83 and 86 and consisted, respectively, of 25 μg of S1-MAb injected intravenously in the tail vein and 50 μg divided equally between intraperitoneal and intravenous routes. Mouse spleen cells were removed for fusion experiments, 3 days after the final injection.

Basic ELISA protocol. Anti-S1 idiotypic hybridomas were identified and characterized by variations of a basic ELISA protocol. To capture appropriate immunological reagents, 96 polyvinyl chloride (PVC) plates with wells (Fisher Scientific Co., Pittsburgh, PA; 14-245-142) were used as the solid phase in the assays.

Modifications of basic ELISA protocol were developed to characterize the antibodies generated in this study. In the basic protocol, wells of PVC plates were coated with 50 μl of an appropriate reagent diluted in PBS. Plates were incubated either for 1 hr at 20 C or overnight at 4 C. All samples were incubated in a humidified box. After coating, wells were emptied and filled with wash buffer (WB) (0.02 sodium phosphate containing 0.015 M NaCl, 0.05% [v/v] Tween 20 [Sigma 1379] and 0.00125% [w/v] thimerosal [Sigma T5125], pH 7.4). The plates were washed 10 times (3 sec per wash, 30 sec total washing time) after this

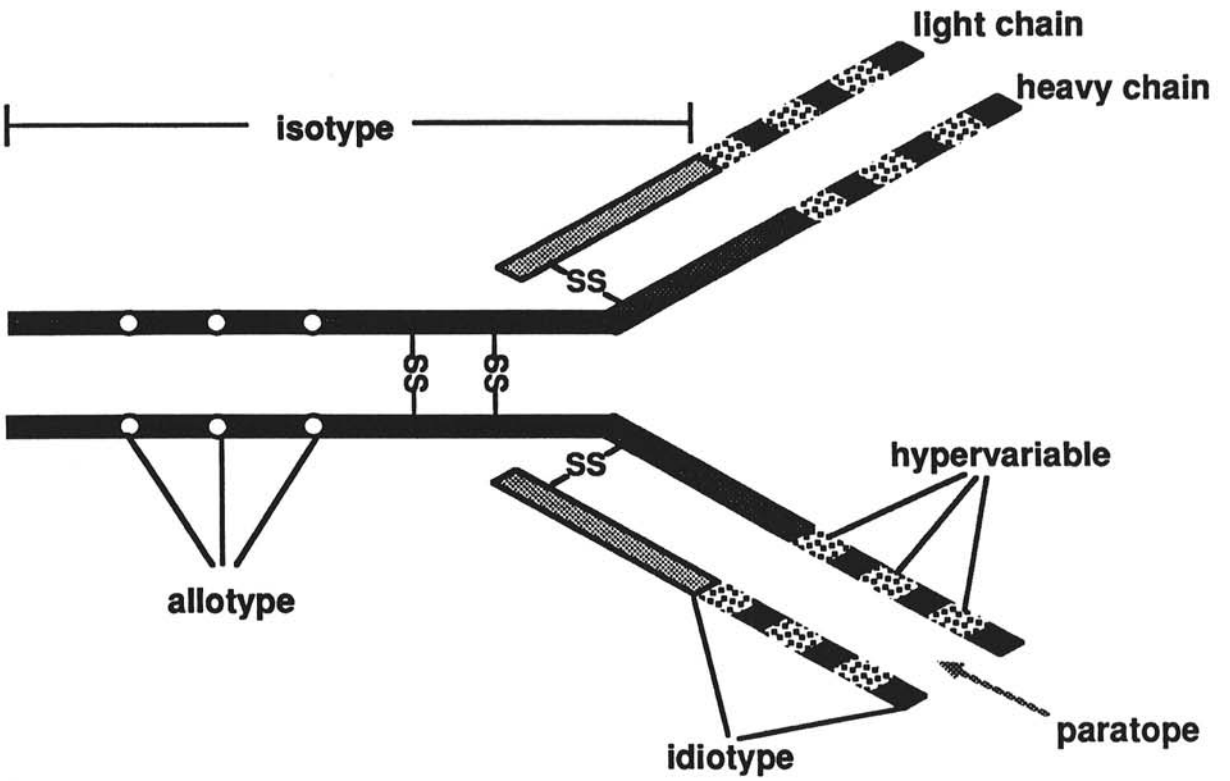

Fig. 1. Diagrammatic representation of an antibody immunoglobulin G molecule.

and every subsequent step. After washing, wells were filled with WB containing 1% bovine serum albumin (BSA; Sigma 4503) and incubated for 1 hr at 20 C. Wells were emptied, and each well was filled with 50 μl of a second immunoreagent diluted in WB. The second immunoreagent contained 1% BSA in WB with the exception of hybridoma medium, which was diluted with an equal volume of WB containing 2% BSA. Plates were incubated for 1 hr at 20 C with virus or for 3.5 hr at 20 C with all other immunoreactants. After washing, 50 μl of alkaline-phosphatase conjugated goat-anti-mouse IgA (Sigma A7659), or IgM (Sigma A7784), or goat-anti-rabbit IgG (Sigma A8025) diluted (1:1,000), or avidin-conjugated alkaline phosphatase (Sigma 2527) diluted

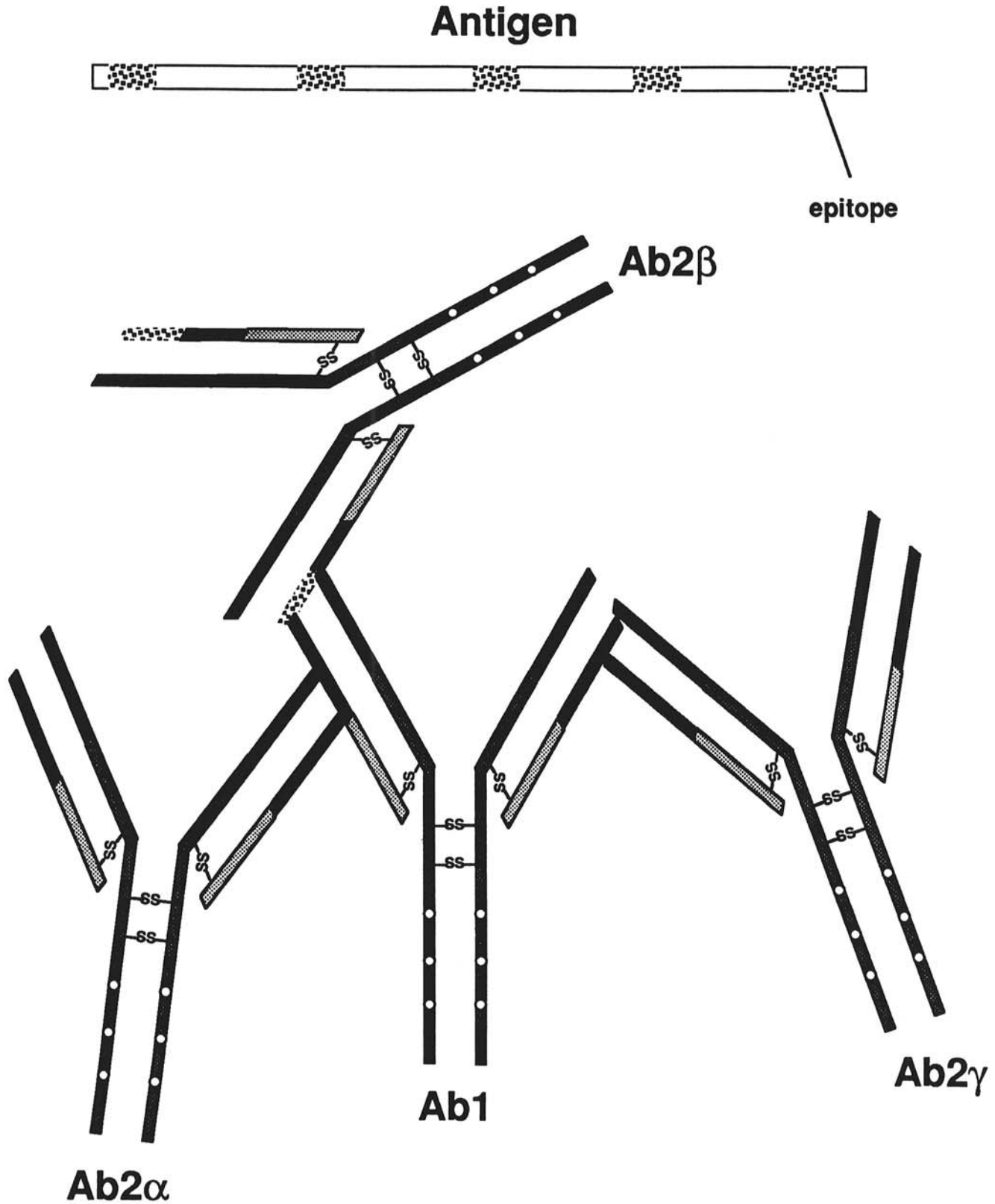

Fig. 2. Diagrammatic representation of interactions that may occur in an antigenically stimulated animal. Anti-idiotypic antibodies are represented as Ab2α (those that recognize idiotopes on the variable region of another antibody are spatially removed from the paratope of that antibody); Ab2β (those that interact with the paratope of another antibody); and Ab2γ (those that recognize idiotopes in or near the paratope of another antibody molecule). Ab1 designates an antibody specific to the immunizing antigen.

to 0.16 μg/ml in WB containing 1% BSA was added to each well. To detect a second mouse IgG antibody bound to S1-MAb-coated wells and which had a different isotype from the S1-IgG antibody, 50 μl of rabbit IgG anti-mouse isotype antibody (Zymed Laboratories Inc., San Francisco, CA; 90-6550), diluted (1:20) in WB containing 1% BSA, was added to each well. The plates were incubated for 1 hr at 20 C and washed, and alkaline-phosphatase conjugated goat-anti-rabbit IgG was added as described. After incubating for 1 hr at 20 C, the plates were washed, and 50 or 100 μl of substrate (*p*-nitrophenyl phosphate, Sigma 104-0, 4.0 mg/ml in 16% [v/v] diethanolamine, pH 9.8) was dispensed into each well. Plates were incubated with substrate at 37 C and reaction products, determined as optical density (OD), were measured with a Dynatech Minireader II (Dynatech Laboratories, Inc., Alexandria, VA) or a Bio-Tek EL 308 (Bio-Tek Instruments, Inc., Burlington, VT) at OD_{405nm}.

In the following procedures, we used modifications of this basic ELISA protocol.

Detection of anti-S1 idiotype antibody production. On day 79, a preliminary screen was made to identify mice that produced anti-S1 idiotype antibody. Wells of a PVC plate (50 microliters per well) were coated for 1 hr at 37 C with sera, diluted (1:10) with PBS, from mice injected with S1-MAb. After washing and blocking, 50 μl of biotinylated S1-MAb (2 μg/ml) was added to each well. The plates were incubated for 1 hr at 20 C and then washed before adding avidin-conjugated alkaline phosphatase. The remainder of the assay followed the basic ELISA protocol. Because some anti-idiotype antibodies have a low affinity for the idiotype-bearing antibody, enzyme reactions were incubated for 18 hr.

Identification of hybridoma anti-idiotypic antibodies by indirect ELISA. A PVC plate was subdivided into three sections, which consisted of four columns of eight wells each. In each section, one column of wells was coated with a control antibody of the same isotype as S1-MAb (MAGII-MAb, 25 ng/well), one column of wells was not coated, and two columns of wells were coated with S1-MAb (25 ng/well). The plates were incubated, washed, and blocked according to the basic ELISA protocol. In each of the three sections, one column of S1-MAb-coated wells received 500 ng of SMV diluted in WB containing 1% BSA. The remaining columns of wells in each section received no additional reagents. The plates were incubated for 1 hr at 20 C, washed, and either stored at 4 C or used immediately.

Hybridoma medium to be assayed for anti-idiotypic antibody production was diluted with an equal volume of WB containing 2% BSA, and 50 μl was transferred to each of four wells that contained either blocking buffer, S1-MAb, S1-MAb, and 500 ng of SMV or MAGII-MAb. A control, conditioned medium (DMEM from a 3- to 4-day-old culture of SP2/0-Ag14 cells), was substituted for hybridoma medium and added to a similar set of four wells of the treatment. The remainder of the assay was completed according to the basic ELISA protocol.

Antibodies that produced generally stronger reactions with S1-MAb than controls that contained blocking buffer, S1-MAb and SMV or MAGII-MAb, were identified and the corresponding cell lines were cloned.

Interaction between soluble anti-idiotypic monoclonal antibodies and bound mouse monoclonal or rabbit polyclonal antibodies. The isotype of putative anti-idiotypic monoclonal antibodies was determined by ELISA by using specific rabbit-anti-mouse immunoglobulins (Zymed Laboratories; 90-6550) as described (6). To assess specificity of putative anti-idiotypic antibodies, wells in PVC microtiter plates were coated with purified S1-MAb, MAGII-MAb, NDV3H4-MAb, or NDV4F11-MAb at 25 ng/well; with purified rabbit polyclonal antibodies raised to SMV, TRSV, or BYDV at 1.0 μg/well; or were left uncoated. The plates were incubated, washed, and blocked according to the basic ELISA protocol. Ascitic fluid, which contained anti-idiotypic monoclonal antibodies, was diluted (1:1,000) in WB containing 1% BSA. Fifty microliters of diluted ascitic fluid was then added to each antibody coated well. Plates were incubated for 3.5 hr at 20 C and washed. Because all antibodies of interest were IgM, 50 μl of goat-anti-mouse IgM conjugated with alkaline phosphatase diluted (1:1,000) in WB containing 1% BSA was added to each well. Assays proceeded according to the basic ELISA protocol.

Interaction between bound S1-MAb and putative anti-idiotypic monoclonal antibodies. To determine if anti-S1-MAb idiotypic antibodies in ascitic fluid interacted specifically with S1-MAb, 50 μl of S1-MAb diluted to 2 μg/ml was added to the first column of a microtiter plate having 12 columns with eight wells each. Twofold dilutions in PBS were made through the 11th column. S1-MAb was not added to the 12th column. The plates were incubated for 1 hr at 20 C, washed, and blocked. Fifty microliters of anti-idiotypic antibody in ascitic fluid was diluted (1:1,000) in WB containing 1% BSA and was added to each well through row 7. No ascitic fluid was added to wells in row 8. Goat-anti-mouse IgM conjugated with alkaline phosphatase was added, and the assay proceeded according to the basic ELISA protocol.

Interaction between soluble anti-SMV polyclonal antibody and bound anti-idiotypic monoclonal antibody in ELISA. If an anti-idiotypic monoclonal antibody could be used in lieu of a viral antigen as a positive control in ELISA, it should capture soluble antibody to viral antigen. Therefore, wells of a PVC microtiter plate were coated with increasing serial dilutions (in PBS) of ascitic fluid produced by a hybridoma designated as 1a or, as a control, purified SMV. A single column of wells was coated with each dilution. MAb produced by hybridoma 1a was selected for further study on the basis of results from previous experiments. After incubation, plates were washed and blocked as described previously. Rabbit anti-SMV polyclonal antibody (0.50 μg/well in WB containing 1% BSA) or anti-TRSV or anti-BYDV polyclonal antibody (0.50 μg/well) was added to appropriate wells in each column. Additionally, a set of wells in one column received no rabbit antibody. The plates were incubated for 1 hr at 20 C and washed. After adding goat-anti-rabbit IgG conjugated with alkaline phosphatase to each well, the assay was completed according to the basic ELISA protocol.

Competition between SMV and rabbit anti-SMV polyclonal antibody for bound MAb 1a anti-idiotypic antibodies. To determine if MAb 1a recognized an idiotope on rabbit polyclonal anti-SMV antibody in close proximity to the anti-SMV antibody paratope, the following competition assay was done.

Wells in PVC plates were coated with anti-idiotypic MAb 1a ascitic fluid diluted (1:1,000) with PBS. Plates were washed and blocked as previously described. Rabbit anti-SMV polyclonal antibody (0.5 or 1.0 μg/well in WB containing 1% BSA) was added to each of eight wells. As a negative control, 50 μl of WB containing 1% BSA was added to a second set of wells. SMV (2 μg/well) diluted with either anti-SMV rabbit polyclonal antibody (0.5 or 1 μg/well in WB containing 1% BSA) or WB containing 1% BSA was added to the first well of each set that contained the reagent corresponding to the diluent. Twofold dilutions were made through the seventh well. No virus was added to the eighth well. The plates were incubated for 3.5 hr at 20 C, washed, and tapped dry. Goat-anti-rabbit IgG conjugated with alkaline phosphatase was then added, and the assay was completed by the basic ELISA protocol.

At each dilution of SMV, competition between SMV and rabbit anti-SMV polyclonal antibody for bound 1a anti-idiotypic antibodies was expressed as percentage of OD_{405nm} inhibition according to the equation, % OD_{405nm} inhibition $= A - B/A \times 100$, in which $A = OD_{405nm}$ of all reactants except SMV, and $B = OD_{405}$ of all reactants.

Competition between S1-MAb and rabbit polyclonal anti-SMV antibodies for bound MAb 1a anti-idiotypic antibodies. Competition between S1-MAb and rabbit anti-SMV polyclonal antibodies for recognition by anti-idiotypic MAb 1a would suggest that these two entities might share a public idiotope. To determine if such an idiotope was present, a protocol similar to that used in the competition assay with SMV, rabbit anti-SMV polyclonal antibody, and anti-idiotypic MAb 1a was followed. The only modification was the substitution of S1-MAb for SMV.

At each dilution of S1-MAb, competition between S1-MAb

and rabbit anti-SMV polyclonal antibody for bound anti-idiotypic MAb 1a was expressed as percentage of OD_{405nm} inhibition according to the equation, % OD_{405nm} inhibition $= C - D/C \times 100$, in which $C = OD_{405nm}$ of reactants with S1-MAb, and $D = OD_{405}$ of reactants without S1-MAb.

Rate study of the bound MAb 1a-soluble anti-SMV rabbit polyclonal antibody interaction. Wells in PVC microtiter plates were coated with 50 μl of MAb 1a diluted (1:2,000) in PBS, washed, and tapped dry. To determine the minimum time for soluble anti-SMV rabbit polyclonal antibody to react with bound MAb 1a, 500 ng of rabbit anti-SMV polyclonal antibody, diluted in WB containing 1% BSA, was added to appropriate wells at 0.5-hr intervals. Three hours after the SMV antibody was first added, the plates were washed and goat-anti-rabbit IgG conjugated with alkaline phosphatase was added. The assay was completed by the basic ELISA protocol.

Interaction between bound MAb 1a and soluble anti-SMV rabbit polyclonal antibody in the presence of leaf sap from uninoculated and SMV-inoculated soybean leaves. Wells in PVC microtiter plates were coated with 50 μl of MAb 1a diluted (1:2,000) in PBS or, for comparison, with 50 μl of MAb S1 diluted to 2 μg/ml, washed, blocked, washed, and tapped dry. Increasing serial dilutions of purified SMV (diluted in either WB containing 1% BSA or in sap from uninoculated soybean leaves [0.5 g of leaf tissue in 10 ml of WB containing 1% BSA]), or sap from inoculated leaves (0.5 g of leaf tissue in 10 ml of WB containing 1% BSA and diluted in sap from uninoculated leaves) were applied to the coated wells. The plates were incubated for 1 hr, washed, tapped dry, and 50 μl of anti-SMV polyclonal antibody (10 μg/ml in WB containing 1% BSA) was added. Goat-anti-rabbit IgG conjugated with alkaline phosphatase was added and the assay was completed by the basic ELISA protocol.

Anti-SMV rabbit polyclonal antibody detection of MAb 1a bound to MAb S1 capture antibodies in the presence of leaf sap from uninoculated soybean leaves. Wells in PVC microtiter plates were coated with 50 μl of MAb S1 (2 μg/ml in PBS), washed, blocked, washed, and tapped dry. MAb 1a in ascitic fluid was diluted (1:10 or 1:50) in sap from uninoculated soybean leaves (prepared as previously described) and 50 μl of the diluted MAb 1a was applied to wells coated or uncoated (negative control) with MAb S1. After incubation at 22 C for 1 hr, plates were washed and 50 μl of biotinylated anti-SMV polyclonal antibody (10 μg/ml in WB containing 1% BSA) was added. After incubation for 1 hr at 22 C and washing, avidin-conjugated alkaline phosphatase was added. The assay was completed by the basic ELISA protocol.

Immunization of rats with MAb 1a ascitic fluid. On day 0, after collecting preimmunization sera, 100 μl of MAb ascitic fluid was diluted with an equal volume of Freund's complete adjuvant and was used to inoculate two Long Evans rats. The rats were bled by cardiac puncture immediately before injection with 0.1 ml of MAb 1a ascitic fluid on days 14 and 25. Rats were exsanguinated on day 35.

Assay for anti-SMV antibodies in rats injected with MAb 1a. Wells in PVC plates were coated with purified SMV (400 ng/well) diluted in PBS for 1 hr at 22 C, washed, blocked, washed, and tapped dry. Ten microliters of rat preimmunization, hyperimmune serum diluted (1:4) in PBS, or PBS was added to SMV-coated wells or to control wells that were only blocked. Plates were incubated at 22 C for 3 hr and then overnight at 4 C, washed, and 50 μl of goat-anti-rat IgG (Sigma 9654) conjugated with alkaline phosphatase was added to each well. The assay proceeded by the basic ELISA protocol.

RESULTS

Immunization of mice with S1-MAb. Only one of the five mice injected with S1-MAb produced serum of sufficient titer to effectively coat microtiter wells and capture S1 antibodies in the presence of other competing serum proteins. The ELISA OD_{405nm} readings for the interaction between the five mouse sera and biotinylated S1-MAb were 0.02, 0.02, 0.04, 0.07, and 0.18 after overnight incubation with substrate. Anti-S1 idiotypic antibodies were generated from the spleen from the last mouse ($OD_{405nm} = 0.18$).

Identification and antibody interaction of hybridomas that produced anti-idiotypic antibodies by indirect ELISA. Anti-S1 idiotypic antibody-producing hybridomas were difficult to identify because differences in OD_{405nm} readings between anti-S1 idiotypic antibodies and S1-MAb or the controls (S1-MAb plus SMV, MAGII-MAb, or blocking buffer) were small (Table 1). However, on the basis of preliminary results, hybridomas 1, 1a, 2, 3, and AB9 were selected for cloning.

Hybridoma 1a produced antibodies that interacted to a greater extent with bound S1-MAb than they did with the control monoclonal antibodies. Antibodies produced by hybridoma 1a also interacted with bound SMV polyclonal antibodies, while antibodies produced by hybridomas 1, 2, 3, or AB9 did not. None of the hybridomas produced antibodies that interacted with bound rabbit TRSV or BYDV polyclonal antibodies or with bound NDV3H4-MAb. Antibodies produced by hybridomas 2 and 3 also interacted with S1-MAb, MAGII-MAb, and NDV4F11-MAb. The interaction between S1-MAb and antibodies from hybridomas 1, 2, 3, and AB9 was inhibited by SMV, whereas the interaction between antibodies from hybridomas 1a and S1 was not.

Anti-S1 idiotypic antibody interactions with bound S1-MAb. The anti-S1 idiotypic antibodies produced by hybridomas 1, 1a, 2, 3, and AB9 reacted specifically with S1-MAb because a decrease in the concentration of antibody used to coat wells corresponded to a decrease in OD_{405nm} readings (Fig. 3). However, differences among anti-idiotypic antibodies with respect to ascitic titer or affinity for S1-MAb were apparent. The data suggested that the order of IgM-antibody titer or affinity of S1-MAb for the anti-idiotypic antibodies was $2 > 1a > 3 > AB9 > 1$.

Interaction between soluble SMV antibody or TRSV antibody and bound anti-S1 idiotypic MAb 1a. ELISA plates were coated with ascitic fluid that contained antibodies produced by hybridoma 1, 1a, 2, 3, or AB9 to capture rabbit anti-SMV polyclonal antibody. Only antibodies produced by hybridoma 1a (optimum dilution of 1:2,000, as selected by calculation of P/N) (14) interacted specifically with rabbit anti-SMV polyclonal antibodies (0.25–1.0 μg/well), but not with control rabbit anti-TRSV (Fig. 4) or anti-BYDV polyclonal antibodies (data not shown). In the same experiment, antibodies from hybridomas 1, 2, 3, or AB9 either did not react with rabbit anti-SMV polyclonal antibodies or did not discriminate rabbit anti-SMV polyclonal

TABLE 1. Interaction of anti-S1 idiotope with antibody S1, other antibodies, and antibody S1 plus soybean mosaic virus (SMV) as measured by ELISA[a]

Coating reagent[b]	Anti-idiotope antibody				
	1	1a	2	3	AB9
S1	0.57	0.51	0.46	1.91	0.13
S1 & SMV	0.41	0.55	0.35	1.75	0.11
MAGII	0.26	0.20	0.20	1.78	0.18
NDV3H4	0.00	0.00	0.00	0.00	0.00
NDV4F11	0.00	0.42	1.09	2.15	0.00
SMV Ab	0.00	0.45	0.00	0.00	0.00
TRSV Ab	0.00	0.00	0.00	0.00	0.00
BYDV Ab	0.00	0.00	0.00	0.00	0.00
WBB	0.13	0.13	0.06	1.45	0.10

[a] Data are $A_{405\ nm}$ values from a representative experiment repeated two or more times.

[b] Designation indicates the immunoreagent used to coat the solid phase: S1 = monoclonal antibody from hybridoma S1 to SMV; MAGII = monoclonal antibody from hybridoma MAGII to maize dwarf mosaic virus, strain A; NDV3H4, NDV4F11 = monoclonal antibodies from hybridomas to Newcastle disease virus; SMV Ab, TRSV Ab, and BYDV Ab = rabbit polyclonal antibodies to soybean mosaic (SMV), tobacco ringspot (TRSV) and barley yellow dwarf (BYDV) viruses, respectively; WBB = 0.02 M sodium phosphate containing 0.15 M NaCl, 0.05% Tween 20, 0.00125% thimerosol, and 1% bovine serum albumin.

antibodies from control antibodies (data not shown). The decrease in OD_{405nm} readings clearly corresponded to a decrease in coating concentration of MAb 1a and showed that the interaction between rabbit anti-SMV polyclonal antibodies and antibodies from hybridoma 1a was specific. The interaction between rabbit anti-SMV polyclonal antibodies and decreasing concentrations of SMV followed a similar pattern in the ELISA (Fig. 4).

Simultaneous competition assay between SMV and rabbit anti-SMV polyclonal antibody for bound MAb 1a. SMV inhibited the interaction between soluble rabbit anti-SMV polyclonal antibody and bound MAb 1a. Inhibition depended on the competing concentration of SMV (Fig. 5A).

Simultaneous competition assay between S1-MAb and rabbit anti-SMV polyclonal antibody for bound MAb 1a. S1-MAb inhibited the interaction of soluble rabbit anti-SMV polyclonal antibody with bound MAb 1a. The inhibition was proportional to S1-MAb concentration (Fig. 5B).

Rate study of the bound MAb 1a-soluble anti-SMV rabbit polyclonal antibody interaction. Incubation time influenced the MAb 1a anti-SMV antibody interaction (Fig. 6). Wells containing 100 μl of substrate gave absorbancy readings approximately 2.5 times greater than wells containing 50 μl of substrate.

Interaction between bound MAb 1a and soluble anti-SMV rabbit polyclonal antibody in the presence of leaf sap from uninoculated and SMV-inoculated soybean leaves. Absorbancy readings increased with increases in the concentration of SMV diluted in WB containing 1% BSA and with increased concentrations of sap from inoculated leaf tissue, but not with purified SMV diluted in sap from uninoculated tissue (Fig. 7A). In those wells in which the capture S1-MAb and the polyclonal anti-SMV

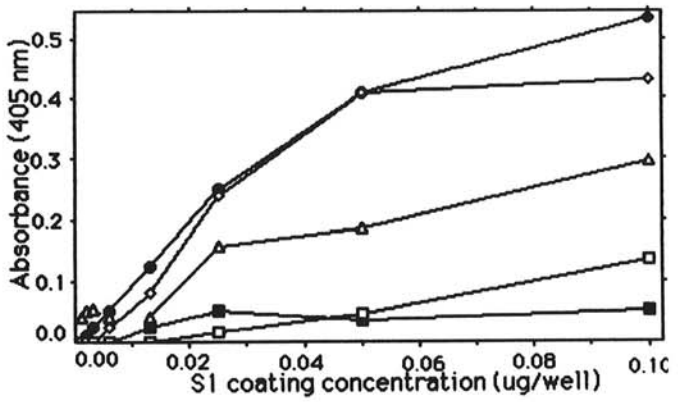

Fig. 3. Interaction of bound antibody from hybridoma S1 with anti-S1 idiotype antibodies 1 (■), 1a (◇), 2 (●), 3 (△), and AB9 (□) as determined by ELISA. Fifty microliters of ascitic fluid produced by each antibody, diluted (1:1,000), was added to a set of wells in microtiter plates coated with increasing concentrations of S1-MAb. Absorbancy readings were made 45 min after the addition of 50 μl of substrate.

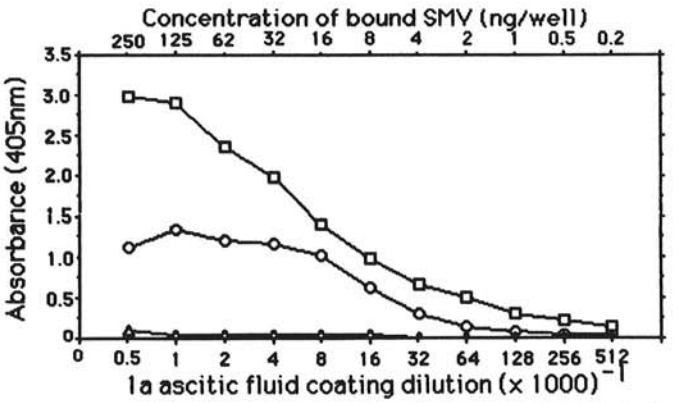

Fig. 4. Interaction of bound anti-S1 idiotypic monoclonal antibody 1a with soluble anti-soybean mosaic virus (○, 0.5 μg/well) or anti-tobacco ringspot virus (△, 0.5 μg/well) rabbit polyclonal antibodies and bound soybean mosaic virus with anti-soybean mosaic virus rabbit polyclonal antibodies (□, 0. 5 μg/well) as measured by ELISA. Data points represent the mean values of seven determinations.

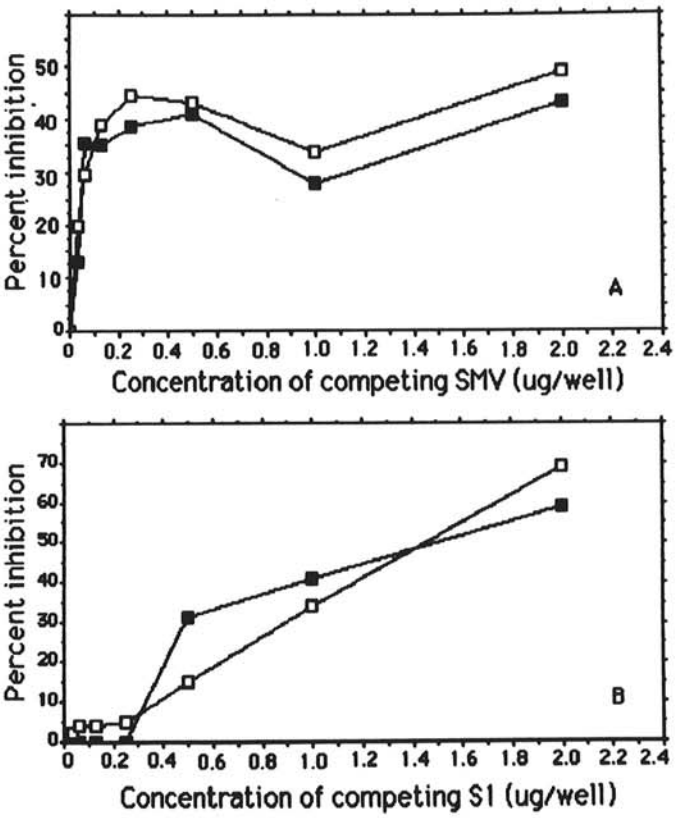

Fig. 5. Results of simultaneous competition assays, as measured by ELISA, between **A,** soybean mosaic virus and rabbit anti-soybean mosaic virus polyclonal antibody; and **B,** between rabbit anti-soybean mosaic virus polyclonal antibody and S1-monoclonal antibody for bound anti-S1 idiotypic monoclonal antibody 1a. Wells in microtiter plates were coated with a (1:1,000) dilution of ascitic fluid of monoclonal antibody 1a. Anti-soybean mosaic virus antibody (■, 0.5 μg; □, 1 μg/well) was added to each well immediately followed by the addition of increasing concentrations of **A,** soybean mosaic virus or **B,** monoclonal antibody S1. Results are expressed as the percentage of inhibition of anti-soybean mosaic virus antibody-monoclonal antibody 1a interaction affected by competing **A,** soybean mosaic virus or **B,** S1-monoclonal antibody. Absorbancy readings were made 0.5 hr after the addition of substrate.

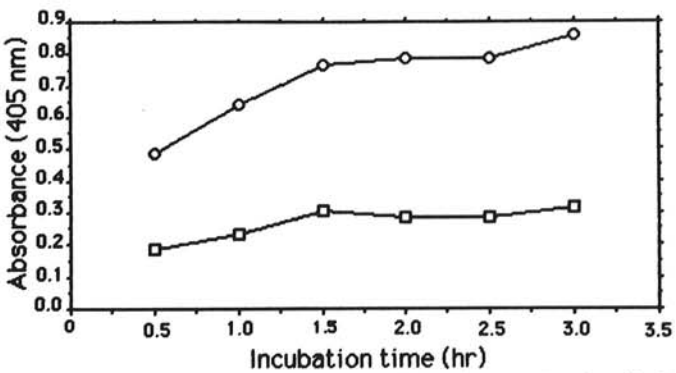

Fig. 6. Rate of interaction of bound anti-S1 idiotypic monoclonal antibody 1a with soluble anti-soybean mosaic virus rabbit polyclonal antibodies as measured by ELISA. Wells in microtiter plates were coated with a (1:2,000) dilution of ascitic fluid of monoclonal antibody 1a. Polyclonal antibodies (0.5 μg/well) were added to appropriate wells at 0.5-hr intervals after coating. Absorbancy readings were measured 1 hr after the addition of substrate (□, 50 μl/well, mean values of six determinations; ○, 100 μl/well, mean values of 14 determinations).

antibody were used, absorbancy readings increased with increased concentrations of purified SMV or sap from inoculated tissue (Fig. 7B).

Anti-SMV rabbit polyclonal antibody detection of MAb 1a bound to MAb S1 capture antibodies in the presence of leaf sap from uninoculated soybean leaves. In ELISA, higher absorbancy readings were obtained from those wells that were coated with MAb S1 followed by the addition of MAb 1a, diluted in leaf sap from uninoculated leaves, and biotinylated polyclonal anti-SMV antibody than from wells to which MAb 1a was not added (Table 2). However, dilutions of MAb 1a exceeding 1:10 yielded absorbancy readings barely above background.

Assay for anti-SMV antibodies in rats immunized with MAb 1a. ELISA, with SMV-coated microtiter wells to detect IgG antibodies in sera from rats injected with MAb 1a, revealed higher absorbancy readings with rat hyperimmune sera than with rat preimmunization sera (Table 3).

DISCUSSION

The purpose of this study was to generate an anti-idiotypic MAb that would bind to microtiter wells to capture SMV antibodies. Idiotypes, which arise during an immune response to an antigen, are themselves antigenic and can elicit an immune response in genetically related animals. Allotype and isotype are features generally shared by antibodies of closely related animals (e.g., BALB/c mice); therefore, inbred animals will not usually respond immunologically to these regions. To avoid generating antibodies to regions outside of the S1 idiotype, we generated anti-idiotypic antibodies by using a syngeneic system whereby BALB/c mice were injected with a BALB/c-derived MAb (S1). It could be argued that our immunization protocol was inefficient because only IgM and not IgG anti-S1 antibodies were obtained. Because mice were stimulated with both glutaraldehyde-fixed S1-producing hybridomas and protein-A purified S1 IgG, it is not possible to determine if fixed S1-producing hybridomas influenced the anti-S1 response. Antigen-mimicking anti-idiotypic antibodies generated as a product formed during an immune response within an individual have been predicted to be, but not exclusively, IgM antibodies of relatively low affinity (9). In a syngeneic system, intact IgG antibodies tend to stimulate only IgM antibody production by interacting with and crosslinking to B-cell receptors. Because a syngeneic system imitates the natural immune response within an individual, it is not surprising that the five anti-idiotypic antibodies characterized in this study were IgM antibodies. A syngeneic reovirus mimicking anti-idiotypic MAb that identifies a cellular reovirus receptor is also an IgM antibody (30).

Sera from mice injected with S1-MAb were used to directly coat microtiter wells to assay for anti-S1-MAb titer. Because serum proteins compete for coating sites on microtiter wells (22), ELISA absorbancy values obtained for the anti-S1-MAb-labeled S1-MAb interaction were predictably low. Because the reaction of protein (e.g., antibodies) in ELISA may react differently in solution phase than in bound phase (38), the assay was designed to reveal those antibodies that, when bound to a microtiter well, would recognize S1-MAb.

The MAb 1a, generated in this study, is recognized by rabbit polyclonal anti-SMV antibody. It also apparently mimics the original SMV antigen because rats injected with ascitic fluid that contained 1a MAb serum generated antibodies to SMV (Table 3). Such antigen mimicry has been previously demonstrated with tobacco mosaic virus (8). Rabbit polyclonal antibodies are often used in ELISA to detect plant viruses; we have demonstrated that an anti-idiotypic antibody can be used to coat microtiter wells in ELISA as a positive control in lieu of a virus to demonstrate the efficacy of antiviral antibodies (Fig. 4). MAb 1a is unlikely to be virus-strain specific. S1-MAb, used to generate MAb 1a, recognizes numerous SMV strains by a variety of tech-

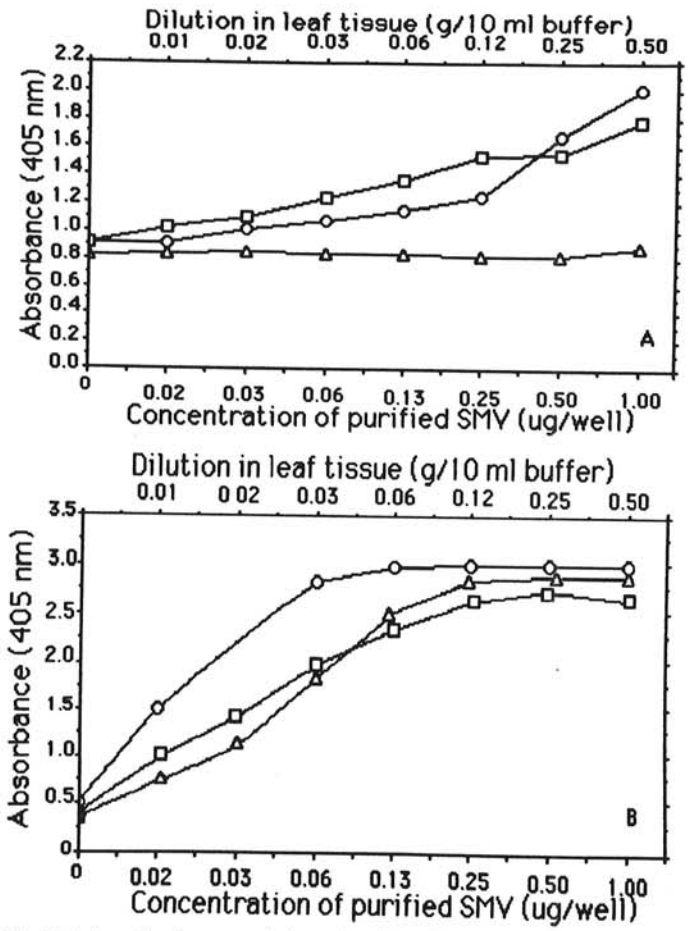

Fig. 7. Interaction between **A,** bound anti-S1 idiotypic monoclonal antibody 1a; or **B,** bound monoclonal antibody S1 with soybean mosaic virus polyclonal antibodies in sap from virus-inoculated soybean leaf tissue diluted in sap from uninoculated soybeans (□), or with purified virus diluted in either 1% bovine serum albumin in wash buffer (○) or in sap from uninoculated soybeans (△). Absorbancy readings were measured 1 hr after the addition of substrate. Data points represent the mean values of 12 determinations.

TABLE 2. Detection of monoclonal antibody (MAb) 1a bound to MAb S1 capture antibodies by anti-SMV rabbit polyclonal antibody in the presence of leaf sap from uninoculated soybean leaves

Dilution of MAb 1a[y]	$A_{405\ nm}$	Standard deviation
1:10	0.248[z]	0.03
1:50	0.025	0.02

[y] MAb 1a was diluted in sap from uninoculated soybean leaves.
[z] Data, minus background, recorded 1 hr after the addition of 100 μl of substrate, are the mean and standard deviations of 10 individual assays. Mean and standard deviations of control wells (containing no S1) were 0.00.

TABLE 3. Detection of anti-soybean mosaic virus antibodies in serum from rats immunized with monoclonal antibody 1a

Rat number	Bleeding number	$A_{405\ nm}$	Standard deviation
1	1 (normal serum)	0.29[a]	0.01
	2	0.37	0.01
	3	0.62	0.01
	4	0.40	0.01
2	1 (normal serum)	0.43	0.02
	2	0.84	0.02
	3	0.48	0.02
	4	0.38	0.02

[a] Data, recorded 20 min after the addition of 50 μl of substrate, are mean values recorded from duplicate experiments. Background values (wells coated with PBS) were subtracted from data shown.

niques (2,13,15). Furthermore, the rabbit polyclonal anti-SMV antibodies recognize all SMV strains (5,31). In addition, ascitic fluid containing 1a MAbs can be added to leaf sap from uninoculated soybean leaves in place of SMV to determine if, in ELISA, the capture antibody (e.g., S1) and the detecting antibody (e.g., SMV polyclonal antibody) are both active (Table 2). However, in this situation, ascitic fluid containing MAb 1a could only be diluted (1:10) to achieve a positive absorbancy reading. Because S1-MAb and anti-SMV compete with each other for binding to MAb 1a (Fig. 5B), it is probable that the number of sites on MAb 1a for S1-MAb and anti-SMV polyclonal antibodies limits, but does not preclude, simultaneous interaction of these antibodies with MAb 1a. The data indicate also that the interaction does not prevent the use of MAb 1a in the assay.

The interaction between soluble anti-SMV polyclonal antibodies and bound MAb 1a was not affected by sap from uninoculated leaves (Fig. 7A). Absorbancy readings increased, however, in the presence of SMV in sap from inoculated leaf tissue or in the presence of purified SMV diluted in WB containing 1% BSA. This suggests that MAb 1a may be an epibody or an anti-idiotypic antibody that interacts with both SMV and the idiotype-bearing SMV antibody (3,7). This is supported by ELISA and western blot analysis data that demonstrate specific interaction between bound SMV and MAb 1a (R. L. Mernaugh, *unpublished results*). The cause for the apparent inhibition between bound MAb 1a and SMV by sap from uninoculated leaves is unclear.

A hybridoma can theoretically produce a limitless supply of antibody. In this study, 50 μl/well of ascitic fluid at a 1:2,000 dilution from hybridoma 1a was nearly optimal for capturing rabbit anti-SMV polyclonal antibody. At this concentration, 5 ml of MAb 1a ascitic fluid could suffice for 200,000 assays without purifying the MAb from ascitic fluid.

Although absorbance increased with incubation for the bound MAb 1a-soluble anti-SMV polyclonal antibody interaction (Fig. 6), a 1-hr incubation period yielded sufficient absorbancy. Because 1 hr is a standard incubation time, this immunoreagent is compatible with many ELISA protocols.

Other anti-idiotypic antibodies have been generated to anti-plant virus antibodies. Rabbit polyclonal IgG anti-idiotypic antibodies have been produced against tobacco mosaic virus (TMV) polyclonal antibodies (8,36) and a BYDV MAb (16). In the TMV system, only about 15% of the polyclonal anti-idiotypic antibodies actually mimicked the antigen (8). The remaining anti-idiotypic antibodies (85%) reacted with idiotopes extraneous to the paratope.

Jerne's immune network theory (17) suggests that an anti-idiotypic antibody may recognize other antigenic structure as well as the idiotope on an antibody molecule. Therefore, although a polyclonal anti-idiotypic preparation may be specific for idiotopes on antibodies, it is possible that those anti-idiotypic antibodies that do not mimic the antigen recognize private idiotopes on other antibodies. These may interact with other miscellaneous antigens to produce misleading results. Furthermore, it is possible that within a population of polyclonal anti-idiotypic antibodies, one or more of the antibodies within the polyclonal anti-idiotypic antibody preparation may bind to and distort the idiotope-bearing antibody molecule (34). If the idiotope-bearing antibody molecule is sufficiently distorted, the antibody will not bind to the antigen (1). Hence, it is possible to erroneously conclude that a polyclonal anti-idiotypic preparation contains a high proportion of antigen-mimicking antibodies when an antigen does not bind with the idiotope-bearing antibody in competition experiments that use putative antigen-mimicking anti-idiotypic antibodies. These reservations led us to generate monoclonal rather than polyclonal anti-idiotypic antibodies. It also suggests why polyclonal BYDV anti-idiotypic antibodies failed to inhibit aphid transmission of BYDV (16).

The antibodies produced by the hybridomas generated in this study are monoclonal anti-idiotypic and not anti-isotypic. This is demonstrated by their inability to interact with IgG2a kappa light-chain antibodies. Furthermore, antibodies produced by some of these hybridomas apparently recognize intraspecies cross-reactive idiotopes shared by monoclonal antibodies from hybridomas S1, MAGII, and NDV4F11 because they all reacted to some extent with these antibodies. This is predicted by the immune network theory, which suggests the presence of intraspecies cross-reactive idiotopes on some antibodies with different antigenic specificities (17).

The antibody produced by hybridoma 1a also recognizes an interspecies cross-reactive idiotope shared by both S1 and SMV antibodies. The cross-reactive idiotope exhibited by these two antibodies must be quite similar as S1-MAb and rabbit anti-SMV polyclonal antibody competed with each other for bound antibody from hybridoma 1a (Fig. 5B). Furthermore, this idiotope on the SMV antibody must be quite close to the SMV antibody paratope because SMV effectively competes with and inhibits the anti-SMV polyclonal antibody-MAb 1a interaction in a simultaneous competition assay (Fig. 5A).

Although not previously demonstrated with antigen-antibody interactions in which a plant pathogen is the antigen, numerous other reports demonstrate interaction between anti-idiotypic antibodies and cross-reactive idiotopes (11,25,28,37). These results suggest that "nonspecific reactions" must be carefully interpreted.

It can be argued that denatured viruses or viral proteins can be used as positive controls instead of an anti-idiotypic antibody. However, they are often incapable of interacting with antiviral antibodies. These results demonstrate that an anti-idiotypic MAb can be a safe, reliable, and inexpensive positive control in assays for exotic pathogens in which quarantines restrict exotic pathogens. They also provide the first example of a monoclonal anti-idiotypic antibody produced against an antibody to a plant virus.

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