Production of Monoclonal Antibodies Against Three Ilarviruses and Alfalfa Mosaic Virus and Their Use in Serotyping

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ABSTRACT

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Fourteen stable hybridoma cell lines that secrete monoclonal antibodies against prunus necrotic ringspot (NRSV), apple mosaic (ApMV), tobacco streak (TSV), or alfalfa mosaic (AMV) viruses were produced by fusing spleen cells of mice immunized with a mixture of all four viruses to mouse myeloma cell lines NS1/1 or P3 X63Ag8.653. Of the seven hybridomas secreting monoclonal anitbody specific for NRSV or ApMV, two were NRSV specific, three were ApMV specific, and two were cross-reactive for some strains of NRSV and ApMV. Five hybridomas secreted antibodies specific for TSV, and two hybridomas secreted antibody specific for AMV. High-titered ascitic fluid was produced to all of the hybridomas and used in indirect ELISA to serotype a panel of isolates of NRSV, ApMV, and TSV. Three serotypes of NRSV, five serotypes of ApMV, and four serotypes of TSV were identified. Titers of hybridoma ascitic fluid (measured by indirect ELISA) ranged from a low of 5,000–62,500 for antibody NA49F8 toward ApMV-F and NRSV-G, to a high of 7,812,000 for N63F10 toward NRSV-G. Only five of the 14 monoclonal antibodies precipitated homologous virus in agar double-diffusion assays. These monoclonal antibodies should prove to be valuable reagents for virus classification and disease diagnosis.

Additional key word: ilarvirus.

Immunological techniques are among the most important tools for the identification, taxonomy, and detection of viruses or virus-infected plants (29). Recent advances in methods, including solid-phase immunoassays (ELISA and RIA) have greatly increased the sensitivity and utility of these immunological tools. However, widespread application of serological techniques is often limited by a lack of readily available, high titered, high quality antisera. This is particularly true for viruses like ilarviruses that are weak immunogens and are difficult to purify in quantities sufficient for antiserum production in rabbits. Even with viruses that are strong immunogens, antisera may vary both quantitatively and qualitatively from animal to animal and among different bleedings from the same animal.

Hybridoma technology introduced by Köhler and Milstein (25) has provided a revolutionary advance in the method of antibody production that eliminates many of the problems associated with conventional antibody production in rabbits. Hybridomas are somatic cell hybrids made by fusing B-lymphocytes of the spleen with myeloma cells. The resulting hybrid cell or "hybridoma" acquires from its spleen cell parent the ability to produce specific antibodies, and acquires from its myeloma cell parent the ability to be cultured indefinitely in vitro. Some advantages to this method of antibody production are: (i) A small quantity of antigen is required (as little as 10 μg) to stimulate the immune response in mice. (ii) All antibody molecules produced by a single hybridoma clone are essentially identical and react with a single antigenic site (monoclonal antibodies). (iii) Hybridoma cells can be preserved indefinitely by freezing in liquid N_2, thus assuring that a continuous supply of antibody can be produced wherever and whenever needed. (iv) Highly specific monoclonal antibodies may reveal serological relationships previously unrecognized with polyclonal sera. (v) Pure antibodies specific for a single antigenic site can be obtained even when impure antigen or antigen mixtures are used as the immunogen (23,24). The specificity of this system requires screening procedures to select only hybridomas that secrete antibody to the desired antigen.

Monoclonal antibodies have been produced to tobacco mosaic virus (1,5,10) and have been used to differentiate several tobamoviruses and to probe the antigenic structure of TMV.

We produced hybridomas that secrete monoclonal antibodies specific for prunus necrotic ringspot (NRSV), apple mosaic (ApMV), tobacco streak (TSV), or alfalfa mosaic virus (AMV). NRSV and ApMV are serologically related (2,4,6,8,11,12) and, together with TSV, are members of the ilarvirus group. AMV and the ilarviruses are biologically, biophysically, and biochemically similar (31). These viruses are multiparticulate, having three or more nucleoprotein particle types differing in size but not density, and a coat protein with a molecular weight of ~25,000 daltons. All four viruses are moderate to poor immunogens in rabbits, often requiring injections twice a week of 1–2 mg of virus per injection for 4–8 wk to produce an antiserum with a double-diffusion titer greater than 1/250 in rabbits (11,12).

The purpose of this study was to determine the utility of monoclonal antibodies to distinguish strains of ilarviruses. Antibodies were titered in indirect ELISA and in agar double-diffusion tests, and immunoglobulin isotypes were determined. Preliminary results have been reported (21,22).

MATERIALS AND METHODS

Virus sources. Prune dwarf virus (PDV), tulare apple mosaic virus (TAMV), AMV, and strains of NRSV, ApMV, and TSV were obtained from donors listed in Table 1. Several isolates of ApMV and NRSV were made from commercial hybrid tea or florabunda roses. ApMV-AB was isolated from *Prunus domestica* 'Ada Bayer' at the USDA facility in Beltsville, MD. All inoculations with virus were done in 0.03 M sodium phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol and 1–2% PVP-10 (Sigma Chemical Co., St. Louis, MO 63178) (18). Isolates of ApMV and NRSV from plum or

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rose were obtained by triturating young leaf or petal tissue in the above buffer and inoculating corundum-dusted cotyledons of *Cucumis sativus* L. 'Lemon.' Single-lesion isolates were maintained in the greenhouse on cucumber or in *Vinca rosea* L. as were other ApMV or NRSV strains.

Purification. NRSV and ApMV were purified from Lemon cucumber 4–5 days after inoculation of the cotyledons with infected cucumber sap. TSV and AMV were purified from leaves of *Datura stramonium* or *Nicotiana tabacum* L. 'Xanthi-nc' 10–14 days after inoculation with either virus. Virus inoculum was extracted from Xanthi-nc 7–14 days after infection with AMV or TSV-WC. All viruses were purified by the hydrated calcium phosphate method (14,20) modified by omitting the acidification step. After the first high speed centrifugation step, viruses were resuspended in 0.05 M tris (pH 7.5) (NRSV and ApMV), 0.03 M EDTA (pH 6.0) (TSV), or 0.03 M tris + 0.01 M NaH_2PO_4 + 0.01 M EDTA (TPE) (pH 7.2) (AMV). Centrifugation through 7–30% sucrose density gradients in the appropriate buffer was used as the final purification step.

Cell lines and medium. The myeloma cell line P3/NS1/1-Ag4-1 (NS1) was kindly provided by M. Lostrom (Fred Hutchinson Cancer Research Center, Seattle, WA 98104). This is a myeloma line that does not secrete immunoglobulin and that produces its own K light chain, but does not produce an immunoglobulin G (IgG) heavy chain (23). Myeloma cell line P3 X63Ag8.653, which does not produce any endogenous K or λ light chains or an IgG heavy chain (23), was used in the second pair of fusions. This cell line was provided by F. Cuttitta (NVI-VA Medical Oncology Branch, Washington Veterans Administration Medical Center, Washington DC). Both myeloma lines were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum, 1 mM pyruvate, 1 mM L-glutamine (glutamine added weekly), and 1% 100 × Pen/Strep (medium and all reagents from Gibco, Grand Island, NY 14072). All myelomas and hybridomas were grown at 37 C in an atmosphere of 7% CO_2. Suspensions of rapidly growing myeloma cells ($<10^5$/ml) were diluted with equal volumes of fresh medium daily for 3 days prior to fusion to ensure that the cells were in optimum condition for fusion (26).

Feeder cells and conditioned media. Peritoneal macrophages were prepared from killed adult BALB/c mice (9). Abdominal skin was removed to expose the peritoneal wall. The wall was bathed in 70% ethanol, and 4–5 ml of 0.34 M sucrose was injected through a 1.024-mm (18-gauge) needle into the peritoneal cavity (care was taken to not puncture the gut). After a gentle massage of the abdomen, the sucrose macrophage solution was withdrawn. Macrophage cells were washed twice in serum-free medium, resuspended in complete medium at 10^5 cells per milliliter, and dispensed (0.1 ml per well) into 96-well tissue culture plates.

Spleen-cell-conditioned medium was made by dispersing the cells of one BALB/c mouse spleen into 100 ml of complete media in a 75-cm^2 tissue culture flask and incubating at 37 C for 24 hr. The medium was used within 3 days of preparation. Fibroblast conditioned medium was used in some of the single-cell cloning procedures and was prepared from complete medium in which a confluent culture in a 75-cm^2 tissue culture flask of MRC-5 human lung fibroblast cells (ATCC CCL 171) had been growing for 24 hr. This fibroblast-conditioned medium was mixed 1:3 with fresh complete medium for hybridoma cloning.

Immunization. Four BALB/c mice were immunized on day 1, by an intraperitoneal (ip) injection of 0.30 ml of a virus mixture in TPE

TABLE 1. Source of virus isolates used in monoclonal antibody production and in virus serotyping studies

Virus	Source	Donor (reference)	Virus	Source	Donor (reference)
NRSV-[a]			ApMV-[a]		
G	Cherry	ATCC PV 22	P	Apple	ATCC PV 32
A	Peach	ATCC PV 295	F	Apple	R. W. Fulton
DPLP	Plum	ATCC PV 34	YB	Yellow birch	A. R. Gotlieb[e] (18)
CH-3	Cherry	G. I. Mink[b]	AB	Plum	E. L. Halk
CH-9	Cherry	G. I. Mink	RMV	Rose	R. W. Fulton (11)
CH-30	Cherry	G. I. Mink	CH	Rose	E. L. Halk
9	Rose	E. L. Halk[c]	50	Rose	E. L. Halk
52	Rose	E. L. Halk	54	Rose	E. L. Halk
130	Rose	E. L. Halk	100	Rose	E. L. Halk
Hop	Hops	C. B. Skotland[b]	215	Rose	E. L. Halk
			Hop	Hops	C. B. Skotland
TSV-[a]					
WC	White clover	ATCC PV 276	PDV[a]	Cherry	ATCC PV 290
B	Soybean	R. W. Fulton[d] (15)	AMV[a]	Alfalfa	ATCC PV 92
U135D	Rose	R. W. Fulton (13)	TAMV[a]	Apple	ATCC PV 265
GV	Grape	R. W. Fulton (15)			
NC	Tobacco	R. W. Fulton (15,17)			

[a] Prunus necrotic ringspot virus (NRSV), apple mosaic virus (ApMV), tobacco streak virus (TSV), prune dwarf virus (PDV), alfalfa mosaic virus (AMV), and tulare apple mosaic virus (TAMV).
[b] Washington State University, IRAEC, Prosser.
[c] Virus isolates from commercial hybrid tea or florabunda roses or *Prunus domestica* 'Ada Bayer,' USDA, Beltsville, MD.
[d] University of Wisconsin, Madison.
[e] University of Vermont, Burlington.

containing NRSV-G (60 μg), ApMV-F (60 μg), TSV-WC (300 μg), and AMV (300 μg) in Freund's incomplete adjuvant. Mice were given an equal dose of virus mix without adjuvant, split between an intravenous (iv) and ip injection on day 120. The spleen cells of two mice were fused on day 123 with the NS1 myeloma cell line. Half the initial immunizing dose of virus mix was injected in the tail vein of the other two mice on day 137, and the spleen cells of these mice were fused with the nonimmunoglobulin-secreting myeloma cell line P3 X63Ag8.653 3 days later.

Cell fusion. Cell fusions were done with polyethylene glycol (PEG) procedures (16,26). Spleen cells were gently dispersed by repeatedly injecting the spleen with serum-free RPMI 1640 (incomplete medium) through a 0.812-mm (20-gauge) needle. Spleen cells (10^8) and myeloma cells (10^7) were washed three times in 40 ml of incomplete medium by centrifugation at 160 *g* for 10 min. Spleen and myeloma cells were combined at a 10:1 ratio and pelleted at 160 *g* for 10 min. The supernatant was aspirated, and the pellet was gently resuspended with 1.5 ml of 40% (w/v) PEG 4000 (Sigma) in serum-free medium added dropwise over a period of 45 sec. The mixture was incubated at 37 C for 75 sec with gentle shaking. The PEG solution was diluted by adding aliquants of 2, 2, and 20 ml of complete medium dropwise over a period of 1 min for each aliquant with a 1-min incubation between aliquants. The cell suspension was centrifuged for 10 min at 160 *g*, and the pellet was resuspended in complete medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT). The fusion product of each spleen was distributed into nine 96-well tissue culture plates pre-seeded with 10^4 peritoneal macrophages in 0.1 ml of complete medium. HAT selective medium supports the growth of spleen-myeloma hybrid cells, but not unfused myeloma cells or myeloma-myeloma cell hybrids (23,24).

Hybridoma plates were examined for cell growth beginning 7 days after the fusion. Cells were fed by replacing 50% of the medium in each well on days 7, 10, 13, and 16 with spleen-cell-conditioned HAT medium, and on days 19, 22, 25, and 28 with spleen-cell-conditioned HT medium (complete medium + 10^{-4} M hypoxanthine + 1.6×10^{-5} M thymidine). Cells were fed every 3–4 days thereafter with complete medium. Hybridomas were screened for antibody production to the immunizing viruses beginning on day 14. Positive hybridomas reaching 50% confluence in a well of a 96-well plate were transfered to 1-ml cultures in a 24-well plate and were retested and then grown in mass culture. Log phase cells from 20–30 ml of medium were pelleted at 160 *g* and resuspended at 10^6–10^7/ml in complete medium containing 10% dimethyl sulfoxide and frozen in 0.5-ml aliquants in liquid N_2.

Cloning. Several hybridoma cell lines were selected for further characterization and were passed two to three times at low cell densities (7), retested for antibody production, and then cloned two to three times to single cells by limiting dilution. Cells were diluted to 20 ml of suspension containing 30–50 viable cells and plated at 0.2 ml per well into a 96-well plate. Single-cell clones were selected from plates in which <35% of the wells contained hybridomas. Cloned cells were grown in either the complete medium or in fibroblast- or spleen-cell-conditioned media. All three systems produced satisfactory results.

Screening hybrid cells. Hybridomas producing antibodies specific for the injected viruses were detected by indirect or modified indirect ELISA (30,32) beginning on day 14. Flexible polyvinyl chloride (Dynatech) 96-well plates were coated with 50 μl/well of purified virus (TSV-WC, NRSV-G, or ApMV-F) at 5 μg/ml in 0.1 M carbonate buffer, pH 9.6. Plates were washed three times with PBS Tween (127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4, and 0.05% Tween-20) and incubated for 20 min with a blocking solution of 1% bovine serum albumin (BSA) in PBS. Hybridoma media at dilutions of 1:5 or greater were made in PBS Tween + 0.10% ovalbumin for assay. Affinity purified goat anti-mouse IgG-IgM alkaline phosphatase conjugate was the detecting antibody (Kirkgard and Perry, Gaithersburg, MD 20879). AMV plates were first coated with rabbit anti-AMV at 2 μg/ml in coating buffer. After the plates were washed in PBS Tween, purified AMV or AMV coat protein was added at 5 μg/ml. This was followed by the hybridoma medium and anti-immunoglobulin conjugate. Direct coating of plates with AMV led to high background readings. Normal mouse sera and immune sera from the mice used for cell fusion were used in the assays for negative and positive controls at dilutions of 1:500–1:1,000. Cell culture medium from normal myeloma cells was used as a negative control for the hybridoma media.

Isotyping and mouse ascites production. Isotyping of monoclonal antibodies was done by agar double-diffusion analysis using subclass specific antisera for mouse IgG1, IgG2a, IgG2b, IgG3, and IgM. Standard myeloma proteins of each isotype were used as positive and negative controls (Litton Bionetics, Charleston, NC 29405). Hybridoma antibody in 20–30 ml of cell culture medium was twice precipitated with 50% saturated ammonium sulfate and resuspended in 2 ml of PBS. Antibody was dialyzed against PBS before loading into wells punched into 0.8% agarose in PBS.

High titered ascitic fluid was produced in pristane-primed BALB/c mice by injecting greater than 5×10^5 hybridoma cells into the peritoneal cavity and then collecting ascitic fluid through a 0.912-mm (19-gauge) needle in 2–3 wk (24).

RESULTS

Immunization. The four mice immunized with 180 μg or less of NRSV-G and ApMV-F and 750 μg or less of TSV-WC and AMV had serum titers (measured by indirect ELISA) of 150,000 for NRSV and ApMV, 400,000 for TSV, and 100,000 for AMV.

Cell fusion and hybridoma selection. Fourteen stable hybridoma clones that secreted monoclonal antibodies specific for NRSV or ApMV (seven clones), TSV (five clones), and AMV (two clones) were produced from the two fusions. In the first fusion the NS1 myeloma cell line was used, and in the second fusion the P3 X63Ag8.653 myeloma cell line was used. In both fusions 50–60 wells of the tissue culture plates contained hybridomas that survived the 2–5 wk required for the cells to reach a population sufficient (50% confluence) for assay. Fifteen hybridomas in fusion 1 and 21 hybridomas in fusion 2 tested positive for antibodies specific for one or more of the injected viruses. However, only four hybridomas from fusion 1 and 19 hybridomas from fusion 2 were still growing well and secreting specific antibodies with continued culturing. The majority of the hybridomas either died or ceased to produce specific antibodies, a characteristic of hybrid cells during the first 6 wk of growth (7,24,26). The surviving hybridomas were passed two or three times at low cell densities and retested for antibody production to select stable hybrids before being cloned two or three times by limiting dilution to single cells.

Cloning. Earlier single-cell cloning was done in spleen-cell- or fibroblast-conditioned media. However, on the second round of cloning one set of 10 cell lines representing hybridomas specific for NRSV, ApMV, TSV, and AMV was split into two groups. Five cell lines were cloned to single cells in fibroblast conditioned medium and five cell lines were cloned in complete medium. A one-third dilution series of log phase cells was made in a column of a 96-well plate (0.20 ml per well) and allowed to settle for 30 min. Wells were then examined under a low-powered objective, and the contents of a well containing 35–50 refractile cells were diluted to 20 ml in the appropriate medium and dispersed into a 96-well plate at 0.20 ml per well. Plates were examined after 7 days for cell growth. In fibroblast conditioned medium 130 of 440 wells, and in the complete medium 123 of 448 wells, contained viable clones. The rest of the cloning was done without conditioned media.

Ascites production and antibody characteristics. One or more subclones of each hybridoma cell line was grown in mass culture and injected back into mice for ascitic induction. Most cell lines produced between 3–8 ml of ascitic fluid per mouse. However, hybridomas T73D9 and T50D8 tended to produce solid tumors and only 1–2 ml of ascitic fluid per mouse, while hybridoma N63F10 represented the other extreme and often produced in excess of 15 ml of ascitic fluid per mouse.

Antibodies or ascitic fluid from the 14 selected hybridoma cell lines were characterized for immunoglobulin isotype, indirect

ELISA titer, agar double-diffusion titer, and reactivity to a panel of virus strains (Tables 2–5). Hybridoma cell lines are identified by the specificity of their monoclonal antibody for NRSV (N), ApMV (A), NRSV and ApMV (NA), TSV (T) or AMV (Al), and by the plate and well number in which they originated. NS1-derived hybrids were from plates 45, 46, 49, and 54. Hybridomas in plates 63, 70, 72, 73, and 74 were derived from myeloma cell line P3 X63Ag8.653.

Seven cell lines selected for cloning and further characterization produced antibodies specific for NRSV or ApMV (Table 2). However, monoclonal antibodies from hybridomas NA70C9 and NA49F8 reacted with both NRSV and ApMV. At least 27 single-cell subclones of hybridoma 70C9 were tested. Twenty-four of the 27 reacted to both NRSV and ApMV, and three subclones lost the ability to produce antibody to either virus. This was also the case with single-cell subclones of NA49F8. This pattern would rule out the possibility that the dual reactivity of the antibody was the result of a mixture of more than one hybridoma, which did occur in plate 74. Hybridomas A74F11 and T74F11 were both derived from the same well in plate 74. However, the first 14 single-cell subclones of hybridoma 74F11 tested segregated into eight TSV-specific and six ApMV-specific hybridomas. Antibody from further single-cell subclones of T74F11 was specific only for TSV and antibody from A74F11 subclones was specific for only ApMV.

ELISA antibody titer of cell culture supernatants ranged from 25 to 100 for NA49F8 toward NRSV-G or ApMV-F to >3,000 for N63F10 for NRSV-G. Antibody titers in ascitic fluid were 100–1,000 times greater than in the cell culture media and ranged from 5,000 to 62,500 for NA49F8 toward ApMV-F and NRSV-G, respectively, to >7,812,000 for N63F10 toward NRSV-G (Tables 2–4).

Five TSV-specific and two AMV-specific hybridomas were produced (Table 3). Both AMV hybridomas had similar properties and were not characterized further.

Monoclonal antibodies from five hybridomas (N63F10, N46E10, NA49F8, T54C5, and T50D8) were capable of precipitating homologous antigen in agar double-diffusion tests (Table 3). With purified virus in the center wall (100 μg/ml) and dilutions of the ascitic fluid in PBS in the six outer wells, precipitin bands formed at, or close to, the antigen well at low dilutions of the ascitic fluid and spiraled out toward the midpoint between antigen and ascitic fluid well at higher dilutions. None of the ApMV- or AMV-specific monoclonal antibodies precipitated purified virus in agar double-diffusion tests.

Serotypes of strains of NRSV and ApMV. Monoclonal antibodies specific for NRSV, ApMV, and TSV were used to screen several isolates of each respective virus to determine if antigenic sites recognized by these antibodies were specific for the immunizing virus strain or were characteristic for several or all strains of the virus (Tables 4 and 5).

Three distinct serotypes of NRSV were identified when nine isolates of NRSV were tested against a panel of seven monoclonal antibodies specific for ApMV or NRSV (Table 4). Strains of NRSV (Table 1) from peach, sweet cherry, or sour cherry included NRSV-G, NRSV-A, and three cherry rugose mosaic strains (CH-3, CH-9, and CH-30). Isolates from commercial rose (NRSV-9, NRSV-52, and NRSV-130) and the Danish plum line pattern strain of NRSV (DPLP) were also examined. Two hybridomas, N46E10 and NA70C9, produced monoclonal antibodies that reacted with all NRSV isolates tested. Serotypes of NRSV were therefore differentiated by the reaction of virus with monoclonal antibodies from hybridomas N63F10 and NA49F8. The Danish plum line pattern strain of NRSV was the only strain of NRSV that did not react with antibody N63F10. None of the NRSV- or ApMV-specific monoclonal antibodies reacted with TSV-WC, AMV, or PDV.

TABLE 2. Hybridoma clones positive to apple mosaic and prunus necrotic ringspot viruses

Clone	Virus	ELISA titer[a]	ID titer[b]	Isotype
A63E10	ApMV-F	625,000	0	IgG1
A74F11	ApMV-F	312,500	0	IgG1
A70A5	ApMV-F	312,500	0	IgG1
NA70C9	NRSV-G	7,812,000	0	IgG2a
	ApMV-F	1,562,000	0	IgG2a
NA49F8	NRSV-G	62,500	320	IgG2a
	ApMV-F	5,000	0	IgG2a
N63F10	NRSV-G	7,812,000	2,560	IgG1
N46E10	NRSV-G	1,562,000	1,280	IgM

[a] Indirect ELISA titer is the reciprocal of the highest dilution of ascites to give an $A_{405\ nm}$ reading >0.10 and a reading greater than three times the background of control mouse sera or ascitic fluid.
[b] Immunodiffusion titer.

TABLE 3. Hybridoma clones positive to tobacco streak or alfalfa mosaic virus

Clone	Virus	ELISA titer[a]	ID titer[b]	Isotype
T54C5	TSV-WC	7,812,000	810	IgM
T74B6	TSV-WC	62,500	0	IgG1
T74F11	TSV-WC	62,500	0	IgG1
T73D9	TSV-WC	312,500	0	IgG1
T50D8	TSV-WC	1,562,000	512	IgG2b
A172B6	AMV	312,500	0	IgG2a
A172D10	AMV	62,500	0	IgG2a

[a] Indirect ELISA titer.
[b] Immunodiffusion titer.

TABLE 4. Reaction of NRSV- and ApMV-specific monoclonal antibodies to strains of NRSV and ApMV in indirect ELISA

Virus strain	Indirect ELISA titer × 10^{-3} to the indicated monoclonal antibody[a]							
	A70A5	A63E10	A74F11	NA49F8	NA70C9	N46E10	N63F10	NM[b]
ApMV-YB	625[a]	625	125	5	1	0	0	0
ApMV-215[c]	125	625	125	0	1	0	0	0
ApMV-AB[d]	625	625	125	0	125	0	0	0
ApMV-P	125	0	0	5	625	0	0	0
ApMV-F[e]	125	625	125	5	625	0	0	0
NRSV-G[f]	0	0	0	12.5	1,562	312.5	7,812	0
NRSV-9[g]	0	0	0	0	1,562	312.5	1,562	0
NRSV-DPLP[h]	0	0	0	12.5	1,562	312.5	0	0

[a] Indirect ELISA titer × 10^{-3} of hybridoma ascitic fluid. A 1:5 dilution series was used with a minimum dilution of 1:500 for NRSV strains and 1:1,000 for ApMV strains.
[b] Normal mouse sera.
[c] A similar reaction pattern was produced by ApMV-100, ApMV-50, ApMV-CH, and ApMV-54.
[d] A similar reaction pattern was produced by ApMV-RMV and NRSV-Hop.
[e] A similar reaction pattern was produced by ApMV-Hop.
[f] A similar reaction pattern was produced by NRSV-CH-9, NRSV-130, and NRSV-A.
[g] A similar reaction pattern was produced by NRSV-CH-3, NRSV-CH-30, and NRSV-52.
[h] DPLP is the Danish plum line pattern strain of NRSV.

Five serological groups of ApMV were revealed when 11 isolates of ApMV (Table 1) were tested against the same panel of monoclonal antibodies used to screen isolates of NRSV (Table 4). ApMV isolates were from apple, rose, hops, plum, and yellow birch. All members of each serological group reacted in a similar manner against a panel of monoclonal antibodies specific for ApMV or NRSV. ApMV-P was the only isolate that failed to react with monoclonal antibodies A74F11 and A63E10. A strong reaction with A70A5, A74F11, and A63E10 was characteristic of all other isolates of ApMV that were tested, but these isolates could be differentiated by their reaction or lack of reaction to NA70C9 or NA49F8 antibodies. Only antibodies from hybridoma A70A5 reacted strongly with all ApMV isolates that were tested.

Serotypes of strains of TSV. TSV strains were differentiated on the basis of their reactions to a panel of five TSV-specific monoclonal antibodies (Table 5). The white clover strain (TSV-WC) and an isolate from rose (TSV-U135D) produced identical reactions to all five of the monoclonal antibodies. TSV strains from soybean (TSV-B), tobacco (TSV-NC), and grapevine (TSV-GV) reacted with only two or three of the TSV-specific monoclonal antibodies. None of the TSV-specific monoclonal antibodies reacted wth NRSV-G, ApMV-F, AMV, TAMV, or PDV.

DISCUSSION

Monoclonal antibodies NA70C9, N46E10, N63F10, and NA49F8 differentiated three serotypes of NRSV and apparently recognized four distinct antigenic differences in NRSV. This conclusion is based on the ability of monoclonal antibodies to precipitate virus in double-diffusion assays (Table 2) and on the reactivity pattern of monoclonal antibodies against a panel of nine NRSV isolates and 12 isolates of ApMV (Table 4). An antigenic site common to all NRSV isolates tested was detected by antibody N46E10, whereas antibody N63F10 reacted with an epitope present on all isolates of NRSV except DPLP. This confirms Fulton's (12) observation that NRSV had a minor population of epitopes not present on DPLP. The determinant reacting with antibody NA70C9 occurred on all nine isolates of NRSV and half of the ApMV isolates tested, although the other half of the isolates of ApMV did react weakly. Antibody NA49F8 recognized an antigenic site present on 5 of 9 NRSV isolates and 4 of 12 ApMV isolates. These two antibodies (NA70C9 and NA49F8) apparently recognized antigenic determinants which may be responsible for at least part of the weak serological relationship reported between some NRSV and ApMV isolates (2,4,6,8,11,12).

Five serotypes of ApMV were identified by monoclonal antibodies NA70C9, NA49F8, A70A5, A63E10, and A74F11. These reactions suggest at least four or five distinct antigenic differences in ApMV (Table 4). Antigenic sites recognized by NA70C9 and NA49F8 are mentioned above. Monoclonal antibody A70A5 was ApMV specific and reacted with a determinant present on all 12 isolates of ApMV that were tested. Antibodies A63E10 and A74F11 are both the same isotype and reacted in a similar manner to all ApMV isolates. It is possible that both antibodies are specific for the same antigenic site and in fact may be identical antibodies arising from independent fusions of cells from the same splenic clone. Alternatively, both antibodies may be specific for separate antigenic determinants common to the 11 isolates of ApMV that were tested. Further analysis by competitive inhibition assays in indirect ELISA and peptide analysis of the immunoglobulins may resolve this question.

Isolates ApMV-Hop and NRSV-Hop from Washington State gave reactions characteristic of ApMV with monoclonal antibodies. NRSV-Hop did have a strong reaction to NA70C9, but did not react with antibody to either N63F10 or N46E10 as did most other isolates of NRSV. Reports from England (2,4) indicate hop isolates serologically resemble either ApMV or an intermediate serotype between ApMV and NRSV. It is possible that isolate NRSV-Hop in addition to its ApMV-specific determinants has antigenic sites characteristic of NRSV strains for which monoclonal antibodies have not yet been produced.

It is interesting that all of the isolates of NRSV and ApMV that were tested could be identified as NRSV or ApMV by reaction with a minimum of two virus-specific monoclonal antibodies. Antibody N46E10 and A70A5 could differentiate all 21 isolates as either NRSV or ApMV. However, it is not known how representative these 21 isolates of NRSV and ApMV are of the serological variations that may exist in nature. It will be interesting to test a larger sample of virus strains to determine if the epitopes recognized by N46E10 and NA70C9 on NRSV, or A70A5 on ApMV occur on all strains of NRSV or ApMV, respectively.

Monoclonal antibodies were able to differentiate serotypes of ApMV on the basis of the presence or absence of one or two antigenic determinants. These minor serological differences were not always apparent or were difficult to interpret by testing with polyclonal sera. Fulton (12) found rose mosaic virus (ApMV-RMV) and ApMV-P to be serologically identical and to cross-react weakly with NRSV antisera in agar double-diffusion tests. ApMV from birch (18,19) and horse chestnut (28) were also serologically indistinguishable from ApMV-P or ApMV-RMV and did not cross-react with antisera to NRSV-G in agar double-diffusion tests. Intermediate serotypes of ApMV and NRSV have been reported from rose (6) and hops (2,4), and it is therefore possible that a continuum of serotypes may exist from NRSV to ApMV. Monoclonal antibodies were able to differentiate ApMV-P, ApMV-RMV, and ApMV-YB, as well as other isolates of ApMV. Both ApMV-P and ApMV-RMV react strongly with the antibody NA70C9 specific to NRSV and ApMV but ApMV-P and ApMV-RMV can be serologically differentiated by reactions with A74F11, A63E10, and NA49F8 antibodies. Isolates of ApMV from commercial rose (ApMV-215, [Table 4]) and the yellow birch isolate (ApMV-YB) reacted very weakly with antibody NA70C9 and thus may be representative of isolates of ApMV less likely to cross-react with antisera to NRSV-G in agar double-diffusion tests. ApMV-215, ApMV-YB, and ApMV-P were further differentiated by reactions to antibodies A74F11, A63E10, and NA49F8.

Monoclonal antibodies from immunization with TSV-WC produced a range of reactions to TSV isolates from white clover, rose, tobacco, soybean, and grape. Only two of them, T50D8 and T54C5, precipitated TSV-WC antigen in double-diffusion assays. These reactions indicate a minimum of four TSV-specific antigenic differences are recognized by antibodies T50D8, T54C5, T73D9, and either T74B6 or T74F11 (Table 5). Antibodies T74B6 and T74F11 are both the same isotype and give the same pattern of reactivity to all TSV isolates tested. Further analysis will be needed to determine if these antibodies are specific for the same antigenic site or not. The serological differences found between TSV-WC, TSV-B, TSV-GV, and TSV-NC with monoclonal antibodies confirm previous reports of serological differences among some of these viruses (14,17). The monoclonal antibodies produced can define these differences in a highly specific and reproducible manner.

Only five of the 14 monoclonal antibodies precipitated homologous virus in agar double-diffusion tests. Nonprecipitating antibodies, however, did have high ELISA titers (Tables 2 and 3). The failure of monoclonal antibodies to precipitate virus could be due to several factors. One possible explanation might be that precipitating antibodies are recognizing antigenic sites on the

TABLE 5. Reaction of TSV-specific monoclonal antibodies to strains of TSV in indirect ELISA

	Indirect ELISA titer $\times 10^{-3}$ to the indicated monoclonal antibody[a]					
TV isolate	T50D8	T54C5	T73D9	T74B6	T74F11	NM[b]
TSV-WC	312.5[a]	312.5	312.5	62.5	62.5	0
TSV-U135D	312.5	312.5	312.5	62.5	62.5	0
TSV-B	0	0	312.5	62.5	62.5	0
TSV-NC	0	0	0	12.5	12.5	0
TSV-GV	2.5	0	12.5	0	0	0

[a] ELISA titer $\times 10^{-3}$. A 1:5 dilution series was used with a minimum dilution of 1:500.

[b] Normal mouse sera or control ascitic fluid.

exterior of the intact virus and therefore are capable of cross-linking virus particles to form a precipitin complex. Nonprecipitating antibodies, on the other hand, may be specific for antigenic sites inaccessible to antibody binding with intact virus. Another possible explanation could be that the conformation of the antigenic site is such that once one arm of an IgG molecule binds, it is physically impossible for the other arm of the antibody molecule to bridge two antigenic sites on different virus particles due either to the distance separating the two antigenic sites or to a limited flexibility of the hinge region of the antibody molecule. In this example, the antibody molecule would be functionally monovalent. One of these appears to be the situation for some of the ApMV-specific monoclonal antibodies (E. L. Halk, *unpublished*). Regardless of the reason why monoclonal antibodies do not precipitate their specific antigen, the fact that many do not may help to explain why it is so difficult to produce high-titered precipitating antisera to ilarviruses in rabbit. If the immune response in rabbit is similar to that in mice, a large population of nonprecipitating antibodies and a low population of precipitating antibodies may be produced. In fact, PDV rabbit antiserum produced at the ATCC had low precipitating titers in agar gel diffusion tests, but at least three orders of magnitude had higher titers in ELISA tests (H. T. Hsu, E. L. Halk, and J. Aebig, *unpublished*). Such sera might be of limited use in agar double-diffusion assays with intact virus, but might be of suitable quality for ELISA or other immunoassays in which all antigenic sites would be measured as a positive response.

Antibody NA70C9 gave a high-titered response (>125,000) to an epitope common to all isolates of NRSV and ApMV that were tested except virus isolates in serotypes represented by ApMV-215 or ApMV-YB (titer = 1,000). Similar low-titered reactions occurred with antibody T50D8 and T73D9 toward TSV-GV relative to TSV-WC. The reduced binding of these antibodies for a specific antigenic site on a heterologous virus strain might result from a partial alteration of the antigenic site. Antigenic structural analyses with monoclonal antibodies directed against TMV, lysozyme, myoglobin, and influenza haemagglutinin (1,3,27,33) indicate that one or two amino acid substitutions in an antigenic site usually result in a substantially reduced binding of specific antibody to that site; however, increased binding also occurs in some cases (1).

These results demonstrate the utility of using monoclonal antibodies to define antigenic relationships among viruses within a taxonomic group and between strains of the same virus. It is doubtful that the seven NRSV- or ApMV-specific and the five TSV-specific monoclonal antibodies produced to ApMV-F, NRSV-G, and TSV-WC represent the total antigenic repertoire of these viruses, but it is interesting that these antibodies have been able to delineate eight serotypes of NRSV and ApMV and four serotypes of TSV. Production of a further group of monoclonal antibodies to a few selected NRSV, ApMV, and TSV strains should provide antibodies specific to a broader range of antigenic determinants among these viruses and allow even greater precision in serological analyses.

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