Preparation and Characterization of Monoclonal Antibodies to Double-Stranded RNA

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ABSTRACT

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Monoclonal antibodies to double-stranded RNA were prepared by fusing splenocytes from BALB/c mice, which had been immunized with poly A · poly U and poly I · poly C, with NS-1 mouse myeloma cells. Of 103 independently derived hybridoma lines which secreted antibodies that reacted with dsRNA, only two competed favorably with rabbit polyclonal antiserum to poly A · poly U for sites on this synthetic RNA. Further characterization of these two monoclonal antibodies showed that they could detect 3 ng poly A · poly U or 10 ng poly I · poly C/ml in indirect double antibody sandwich enzyme-linked immunosorbent assay tests. The antibodies did not react with double-stranded DNA, rRNA, or tRNA. They could distinguish between total nucleic extracts from virus-infected and uninfected plants in some but not all cases.

Since it was realized that single-stranded RNA plant viruses replicate through a double-stranded (ds) RNA intermediate (3), there has been significant interest in using this dsRNA as a basis for diagnosis. The most successful application of this idea has been to combine rapid nucleic acid extraction and partial purification of dsRNA with polyacrylamide gel electrophoresis (4). This procedure not only determines the presence or absence of most plant viruses, but often can be used to identify the virus. Conclusions based on the presence of dsRNA make the assumption that uninfected plants do not contain dsRNAs with molecular weights similar to the replicative forms of plant viruses. The widespread use of dsRNA analysis for preliminary virus diagnosis attests that this assumption is usually, but not always (2), valid.

Double-stranded RNAs can elicit specific antibodies in rabbits (9). There have been some successes using these antibodies as

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a substitute for gel electrophoresis to detect dsRNAs (7,10), but application of immunoassays for dsRNA has been limited because of the low titer and low affinity of antisera, poor binding of dsRNA to solid supports, and concerns about background reaction with low molecular weight dsRNAs in healthy plants. Possibly some of these drawbacks can be overcome by producing an antibody with greater affinity to dsRNA in a higher titer than that in polyclonal antisera. This report describes production of monoclonal antibodies to dsRNA and their evaluation.

MATERIALS AND METHODS

Production and screening of hybridomas. Two BALB/c mice were immunized by intraperitoneal injection with 0.5 ml of 1 mg/ml polyadenylic-polyuridylic acid (poly A · poly U) (minimum mol. wt. = 100,000) (Sigma Chemical Co., St. Louis, MO) and 1 mg/ml polyinosinic-polycytidylic acid (poly I · poly C) (minimum mol. wt. = 100,000) (Sigma Chemical Co.) in phosphate buffered saline (PBS; 0.15 M sodium chloride, 0.015 M sodium phosphate, pH 7.0) mixed with 0.5 ml of Freund's complete adjuvant on days 1, 14, 28, 56, and 70. On day 84 the mice were boosted with a 0.1 ml intravenal injection containing 0.1 mg/ml poly A · poly U and 0.1 mg/ml poly I · poly C in PBS. On day 87 the mice were sacrificed by decapitation and the blood was collected. Spleens were harvested, processed, and fused with NS-1 myeloma cells as described previously (6). The fused cells were diluted to a volume of 1,500 ml of HAT medium (Hb102 medium (Hana Biologics, Berkeley, CA) containing 1 mM sodium pyruvate, 500 μg/ml L-glutamine, 34 μg/ml hypoxanthine, 9.7 μg/ml thymidine, 0.36 μg/ml ominopterin, 20% fetal calf serum, and 30 μg/ml gentamycin) and seeded in 96 well microtiter plates.

Culture fluids from wells with single or multiple colonies were screened by indirect double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 10 μg/ml (A_{280nm} = 0.014) rabbit polyclonal inmunoglobulins to poly A · poly U in 0.05 M sodium carbonate buffer, pH 9.6, and placed at 4 C overnight. These polyclonal antibodies were prepared in 1981 using described procedures (7). They reacted with dsRNA but not with dsDNA or ssRNA in double diffusion or DAS ELISA tests. After washing three times in PBS-Tween-PVP buffer (PBS containing 0.05% Tween 20 and 2% polyvinylpyrrolidone), 0.25 ml of 30 ug/ml poly A · poly U plus 30 ug/ml poly I · poly C in PBS-Tween-PVP was added to each well of each plate and incubated for 4 hr at 37 C. After washing, culture fluid was added to each well and incubated overnight at 4 C. The next morning, after washing, a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma Chemical Co.) was added and incubated for 4 hr at 37 C. After washing, the presence of dsRNA-specific mouse antibody was determined by adding 1 mg/ml of *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8. Cells in wells testing positive for production of antibody to dsRNA were subcloned twice by limiting dilution and then retested.

The culture fluids from positive cell lines were typed with a Mouse Typer Isotyping Kit (Bio-Rad Laboratories, Richmond, CA), concentrated 20-fold by ammonium sulfate precipitation, purified by affinity chromatography with Affi-Gel Blue (Bio-Rad Laboratories), and tested for their ability to compete with rabbit polyclonal antibody to poly A · poly U. In this assay, indirect DAS ELISA was performed as above except the antigen consisted of 1.0 μg/ml poly A · poly U plus 1.0 μg/ml poly I · poly C, and the test (second) antibody consisted of 10 μg/ml rabbit polyclonal immunoglobulins to poly A · poly U mixed with 10 ug/ml mouse monoclonal immunoglobulins. This assay was not intended to precisely compare affinities but to indicate which cell lines might be most suitable for further studies.

Ascites to two cell lines were produced by injecting the cells, grown to high concentration, from one each of T75 flasks into pristane-primed BALB/c mice intraperitoneally. After 7–10 days, the ascites fluid was removed from the abdomens of the mice every three days until they died. IgG was purified from the ascites fluid by affinity chromatography (Affi-Gel Blue, Bio-Rad Laboratories).

Ouchterlony double-diffusion assay. Ouchterlony double-diffusion assay was performed as described (8) in 5 cm-diameter plastic disposable petri plates containing 8 ml of 0.8% Ionagar in PBS. Wells 4 mm in diameter were cut at the corners and center of a regular hexagon 42 mm in circumference. The center well contained 0.02 ml of 1 mg/ml poly A · poly U in PBS. The peripheral wells contained 0.02 ml of crude polyclonal antibodies to poly A · poly U from two rabbits, crude mouse polyclonal antibody to a mixture of poly A · poly U and poly I · poly C, monoclonal antibodies (crude ascites) to a mixture of poly A · poly U and poly I · poly C from two cell lines, or a monoclonal antibody (crude ascites, IgG2b) to tomato ringspot virus (TmRSV).

Determination of antibody specificity. The specificity of two selected monoclonal antibodies was determined by indirect DAS ELISA as described above for the initial screening of culture fluids and by plate-trapped antigen indirect ELISA (5). In plate-trapped ELISA, the plates were coated with antigen followed sequentially by rabbit or mouse antibody, goat anti-rabbit or anti-mouse antibody, and substrate. The antigens were 1.0 μg/ml of poly A · poly U, poly I · poly C, brome mosaic virus (BMV) replicative form RNA, calf thymus DNA, methylated phage lambda DNA, yeast rRNA, or yeast tRNA. All antigens were purchased from Sigma Chemical Company, except BMV

TABLE 1. Specificity of antibodies to double-stranded RNA as determined by indirect double antibody sandwich enzyme-linked immunosorbent assay[a]

	Antibody[b]	
Antigen	MAb-2	MAb-3
poly A · poly U	1.490	1.582
poly I · poly C	0.679	0.600
BMV[c] replicative form RNA	0.898	0.756
calf thymus DNA	0.020	0.050
methylated lambda DNA	0.047	0.037
yeast rRNA	0.079	0.045
yeast tRNA	0.048	0.059

[a]Each treatment consisted of coating 10 wells of a microtiter plate with 10 μg/ml of rabbit polyclonal IgG to poly A · poly U, followed sequentially by 1.0 μg/ml of antigen, 10 μg/ml of anti-dsRNA ascites, a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody, and substrate.

[b]Mean optical densities (A_{405nm}) from the 10 measurements. The reaction time was 30 minutes. Standard deviations ranged from 0.003 to 0.157.

[c]BMV = brome mosaic virus.

TABLE 2. Specificity of antibodies to double-stranded RNA as determined by plate-trapped antigen enzyme-linked immunosorbent assay[a]

	Antibody[b]		
Antigen	MAb-2	MAb-3	Rabbit polyclonal Ab
poly A · poly U	0.882	0.894	1.158
poly I · poly C	0.707	0.781	0.966
BMV[c] replicative form RNA	0.739	0.781	0.911
calf thymus DNA	0.056	0.024	0.068
methylated lambda DNA	0.063	0.018	0.057
yeast rRNA	0.056	0.059	0.048
yeast tRNA	0.047	0.021	0.036

[a]Each treatment consisted of coating 10 wells of a microtiter plate with 1.0 μg/ml of antigen, followed sequentially by 10 μg/ml of antibody to dsRNA, a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibody, and substrate.

[b]Mean optical densities (A_{405nm}) from 10 measurements. The reaction time was 30 minutes. Standard deviations ranged from 0.001 to 0.132.

[c]BMV = brome mosaic virus.

replicative form RNA, which was provided by Dr. Fred Gildow, Pennsylvania State University.

Determination of antibody sensitivity. Antibody sensitivity was determined by indirect DAS ELISA as described above for the initial screening of culture fluids. The first (trapping) antibody was rabbit polyclonal antibody to poly A · poly U. The antigens were concentrations of 100, 30, 10, 3, 1, and 0 ng/ml of poly A · poly U or poly I · poly C. The second (detecting) antibody was 10 μg/ml of purified ascites from cell lines 2 or 3 or purified IgG2b to TmRSV.

Extraction and testing of dsRNAs from tissue. Double-stranded RNAs were extracted by Dr. Fred Gildow using method I of Morris and Dodds (4). The tissues were 100 μg of *Rhopalosiphum padi* infected with Rhopalosiphum padi virus (RhpV) (1), or 5 g of uninfected *R. padi*, barley infected with BMV, tobacco infected with tobacco mosaic virus (TMV), cucumber infected with cucumber mosaic virus (CMV), barley containing an uncharacterized high molecular weight dsRNA, wheat infected with wheat spindle streak mosaic virus (WSSMV), uninfected barley, uninfected wheat, and uninfected cucumber. Ethanol precipitates were dissolved in 0.6 ml of PBS-Tween-PVP and used as the antigen in indirect DAS ELISA as described for the initial screening of culture fluids. The coating antibody was 10 μg/ml rabbit polyclonal antibody to poly A · poly U, and the second antibody was 10 μg/ml purified antibody from cell line 2 ascites. A portion of each sample was also examined by polyacrylamide gel electrophoresis (4).

RESULTS

The fusion of NS-1 myeloma cells with spleens of BALB/c mice immunized with a mixture of poly A · poly U and poly I · poly C resulted in over 5,000 microtiter plate wells containing HAT-resistant cells. Culture fluid from 103 of these wells reacted with a mixture of poly A · poly U and poly I · poly C by indirect DAS ELISA. Mixing rabbit polyclonal antibody to poly A · poly U with each of the 103 purified mouse monoclonal antibodies reduced the absorbance by at least 90% in 101 cases, indicating that the polyclonal antibody bound more effectively to the poly A · poly U than all but two of the monoclonal antibodies. The other two monoclonal antibodies, hereafter referred to a MAb-2 and MAb-3, which competed much better with polyclonal antibodies for sites on the poly A · poly U (the absorbance was reduced by 62% and 64%, respectively), were selected for further study. MAb-2 and MAb-3 were both of the IgG2b subclass based on reaction with IgG subclass specific antibody (Bio-Rad Laboratories).

Neither MAb-2 nor MAb-3 precipitated poly A · poly U in an Ouchterlony double-diffusion assay. A mouse polyclonal serum pooled from the mice that were used to generate the MAbs also did not precipitate the synthetic dsRNA. The positive controls, polyclonal antibody to poly A · poly U from two different rabbits, both produced precipitin lines.

Both MAb-2 and Mab-3 had general specificity for dsRNA either when the antigens were trapped by rabbit polyclonal antibody to dsRNA (Table 1) or by the plastic plate (Table 2). Neither of the antibodies reacted with calf thymus DNA, methylated lamda DNA, yeast rRNA, or yeast tRNA.

Both monoclonal antibodies would detect 3 ng/ml of poly A · poly U or 10 ng of poly I · poly C in an indirect DAS ELISA (Table 3). A control IgG2b MAb to TmRSV did not react with any concentration of poly A · poly U or poly I · poly C.

MAb-2, used as the second antibody in indirect DAS ELISA, clearly detected dsRNA in partially purified dsRNA extracts from *R. padi* infected with the single-stranded RNA aphid virus RhpV, barley infected with BMV, tobacco infected with TMV, cucumber infected with CMV, and barley containing a high molecular weight dsRNA of unknown origin (Table 4). Extracts from wheat infected with WSSMV gave only slightly higher absorbance values than equivalent extracts from uninfected wheat. Double-stranded RNA extracts from uninfected barley, wheat, tobacco, or cucumber gave much higher values than extracts from uninfected aphids or buffer. Polyacrylamide gels of the same extracts showed strong dsRNA bands for all of the extracts that were positive using MAb-2, except for TMV-infected tobacco, which gave a faint band in the gels (ethidium bromide stained). No bands were visible for extracts from healthy barley, wheat, tobacco, or cucumber or WSSMV-infected wheat.

TABLE 3. Sensitivity of monoclonal antibodies to double-stranded RNA[a]

		Antigen concentration (ng/ml)[c]					
Antigen	Antibody[b]	100	30	10	3	1	0
poly A · poly U	2	0.943	0.489	0.256	0.134	0.045	0.054
poly I · poly C	2	0.552	0.380	0.161	0.082	0.072	0.011
poly A · poly U	3	0.959	0.493	0.203	0.166	0.083	0.014
poly I · poly C	3	0.528	0.348	0.143	0.035	0.018	0.015
poly A · poly U	Tm	0.021	0.035	0.020	0.022	0.013	0.017
poly I · poly C	Tm	0.024	0.030	0.010	0.032	0.012	0.019

[a]Each test consisted of coating 10 wells of a microtiter plate with 10 μg/ml of rabbit polyclonal IgG to poly A · poly U, followed sequentially by various concentrations of antigen, 10 μg/ml of MAb, a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody, and substrate.

[b]MAb-2 to dsRNA from cell line 2; MAb-3 to dsRNA from cell line 3; MAb-Tm to tomato ringspot virus.

[c]Mean optical densities (A_{405nm}) from the 10 measurements. The reaction time was 30 minutes.

DISCUSSION

Monoclonal antibodies were produced which specifically reacted with double-stranded RNA and not with single-stranded RNA, tRNA, or double-stranded DNA. These antibodies can be used in conjunction with polyclonal antibodies to dsRNA to detect replication intermediates of several viruses in extracts from infected plants or insects by indirect DAS ELISA. These antibodies may be useful for screening large numbers of samples for the presence of viruses by replacing the electrophoresis step of the nucleic acid analysis.

The specificity of the two monoclonal antibodies was determined using both indirect DAS ELISA, in which the antigens were first trapped by rabbit polyclonal antisera, and by a plate-trapped antigen ELISA. It cannot be ruled out that the observed

TABLE 4. Analysis of extracts from various tissues for double-stranded RNA using antibodies to double-stranded RNA[a]

Tissue	Expt. 1[b]	Expt. 2[b]	Expt. 3[b]
RhpV[c]-infected *Rhopalosiphum padi*	1.166	1.768	1.292
Uninfected *R. padi*	0.062	0.068	0.003
BMV[d]-infected barley	0.727	0.749	0.693
TMV[e]-infected tobacco	0.467	0.337	0.419
CMV[f]-infected cucumber	0.853	0.621	0.735
Barley containing an uncharacterized dsRNA	0.904	1.883	0.999
WSSMV[g]-infected wheat	0.383	0.294	0.325
Uninfected barley	0.209	0.220	0.207
Uninfected tobacco	0.181	0.191	0.134
Uninfected cucumber	0.292	0.294	0.260
Uninfected wheat	0.230	0.266	0.254
Buffer	0.088	0.098	0.001

[a]Each treatment consisted of coating 10 wells of a microtiter plate with 10 μg/ml of rabbit polyclonal anti-poly A · poly U, followed sequentially by dsRNA extracts from the various tissues, 10 μg/ml of monoclonal antibody 2 from ascites, a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody, and substrate.

[b]Mean optical densities from the 10 measurements. The reaction time was 30 minutes. Standard deviations for the treatments (excluding the buffer treatment) ranged from 0.009 to 0.119.

[c]RhpV = Rhopalosiphum padi virus.

[d]BMV = Brome mosaic virus.

[e]TMV = Tobacco mosaic virus.

[f]CMV = Cucumber mosaic virus.

[g]WSSMV = Wheat spindle streak mosaic virus.

specificity is due to antigen trapping rather than reaction with the monoclonal antibodies. For example, it is likely that tRNA is not trapped by the rabbit polyclonal antibody and possible that it is not trapped by the plastic plate. However, it is known that all the nucleic acids listed in Table 1, when labeled using photobiotin, will bind to microtiter plates under the coating conditions used (Powell, *unpublished*). From a practical standpoint, whether the specificity of the assay is due to the coating antibody, the monoclonal antibody, or both, is not important for indirect DAS ELISA.

The monoclonal and polyclonal antibodies gave higher absorbance readings with poly A · poly U than poly I · poly C. The differences, although not large, were reproducible. Again, it cannot be ruled out that poly A · poly U has a greater affinity for plastic than poly I · poly C. Competition experiments with labeled nucleic acids have not been performed.

The indirect DAS ELISA detected 3 ng/ml of poly A · poly U and 10 ng/ml of poly I · poly C. This compares favorably with detection of dsRNAs by gel electrophoresis in which 10 ng can be detected by silver staining. Double-stranded RNA extracts from TMV-infected tobacco which gave faint bands on Ethidium-bromide-stained polyacrylamide gels were clearly positive using the indirect DAS ELISA (Table 4).

The utility of the monoclonal antibodies was in some ways disappointing. The use of indirect DAS ELISA did not eliminate high background in dsRNA extracts from apparently healthy plants. The background was even higher in impure extracts (crude sap), precluding its use as an antigen source. The source of this background is unknown. There were no bands visible in polyacrylamide gels of dsRNA extracts from any of the healthy tissue when stained with ethidium bromide or silver. The antibody to dsRNA may be reacting with nucleic acids which are either too small or too heterogeneous to be resolved on the gels. Most of the background could be removed by treatment with ribonuclease A in low, but not high, salt.

In summary, the monoclonal antibodies prepared to dsRNA react specifically with all dsRNAs tested (poly A · poly U, poly I · poly C, and dsRNA) and will differentiate between plants infected with BMV, TMV, or CMV and healthy plants. An indirect dsRNA ELISA using these antibodies should be useful in certain applications for initially screening plants or insects for viruses.

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