Murine Monoclonal Antibodies Produced Against Two Illinois Strains of Barley Yellow Dwarf Virus: Production and Use for Virus Detection

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ABSTRACT

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A hybridoma cell line that secretes antibodies produced against an Illinois strain of barley yellow dwarf virus (BYDV) transmitted nonspecifically by *Rhopalosiphum padi* and *Sitobion avenae* (BYDV-PAV-IL) and two lines that secrete antibodies produced against an Illinois strain transmitted specifically by *R. padi* (BYDV-RPV-IL) were produced by somatic cell fusion between mouse myeloma cell line SP2/0-Ag14 and spleenocytes from BALB/c mice immunized with either virus strain. Ascitic fluid produced by clones PAV-IL-1, RPV-IL-1, and RPV-IL-5 had titers of 10^8, 10^8, and 10^9, respectively, in triple antibody sandwich enzyme-linked immumosorbent assay (TAS-ELISA) with sap extracted from virus-infected plants. All three ascitic fluids had titers of 10^3 in ELISA with microtiter plates coated at pH 9.6 with purified virus for 2 hr at 37 C. Immunoglobulin subclasses were IgG1 for PAV-IL-1 and RPV-IL-1 and IgG2a for RPV-IL-5, all with kappa light chains. Each of the antibodies was used sucessfully to trap homologous virus in double-antibody sandwich ELISA. In TAS-ELISA, clone PAV-IL-1 detected 17 of 17 PAV-like isolates in dried tissue from four U.S. states and 10 other countries. Clones RPV-IL-1 and RPV-IL-5 both detected seven of seven RPV-like isolates in dried tissue from three states and three other countries. None of the clones detected any of 13 other BYDV isolates tested.

Barley yellow dwarf (BYD) is caused by several virus strains which were differentiated originally by vector specificity (10,22). On the bases of cytopathology (11), serology (1,23), dsRNA profile (9), and nucleic acid hybridization (3,26), the strains have been divided into groups I and II by the International Committee on Taxonomy of Viruses (18) and into two viruses in the *Description of Plant Viruses* by the Association of Applied Biologists (27). Two strains of barley yellow dwarf virus (BYDV) have been studied in Illinois: a vector-nonspecific strain transmitted by both *Rhopalosiphum padi* L. and *Sitobion* (*Macrosiphum*) *avenae* Fabr., designated BYDV-PAV-IL, and a strain transmitted specifically by *R. padi*, designated BYDV-RPV-IL. BYDV-PAV-IL is a member of BYD group I, and BYDV-RPV-IL is a member of group II.

BYD viruses belong to the luteovirus group of plant viruses (27). Luteoviruses occur in low concentrations in host plant tissue and are difficult to purify, probably because they are phloem limited. For these reasons, production of antiserum to BYDV strains has been difficult, and the worldwide supply is limited. Monoclonal antibodies provide a potentially unlimited supply of uniform antibody of defined specificity. Monoclonal antibodies

to some strains of BYDV have been produced and used for virus detection (8,14,21,24). However, none of the monoclonal antibodies produced to BYDV strains from North America is currently widely available. The objective of this research was to produce hybridomas secreting monoclonal antibodies useful for detection of BYDV strains and for examination of relationships among luteoviruses. The latter aspect is reported elsewhere (7).

MATERIALS AND METHODS

Viruses and purification. The virus strains used for immunization were BYDV-PAV-IL (6,12) and BYDV-RPV-IL (19,20). Purification procedures were as previously described (6,20). The origins and designations of BYDV strains and isolates used to test the specificity of cloned monoclonal antibodies are listed in Tables 1–3.

Cell lines and media. SP2/0-Ag14, a non-immunoglobulin-secreting mouse myeloma, was cultured in Dulbecco's Modified Eagle's Medium containing L-glutamine (Sigma Chemical Co., St. Louis, MO) with 4,500 mg/L of glucose and 10% fetal bovine serum (Cibco, Grand Island, NY) as a base medium, containing 20 μg/ml of 8-azaquanine (Sigma Chemical Co.), for 10 days before lymphocyte cell fusion. Cells were cultured in a Steri-Cult Incubator (Forma Scientific, Marietta, OH) which maintains 7.2% CO_2 in an air, 98% humid, 37 C atmosphere.

Production of hybridomas. Female BALB/c mice 8–9 wk old were immunized by intraperitoneal injection with 45–75 μg of purified virus at each injection. Injection volumes were 100 μl of a 1:1 emulsification of virus in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) for the primary injection and incomplete Freund's adjuvant for the following two booster injections. Immunizations were at intervals of 3–4 wk. Blood samples were collected 5 wk after the first infection by a retro-orbital venous plexus bleed using a 100-μl disposable micropette. The blood was allowed to clot at 4 C and centrifuged, and the sera were screened for specific circulating antibody using a triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA). Mice that were producing virus-specific antibodies were sacrificed and asceptically splenectomized, and spleenocytes were isolated for use in polyethylene glycol-mediated lymphocyte cell fusion (15). Fused cells were plated in HAT medium, which contains hypoxanthine, aminopterin, and thymidine, as per Kennett et al (15) in 24-well sterile culture plates (Corning Glass Works, Corning, NY) and grown at 7.2% CO_2 in air atmosphere. After 2–3 wk, HAT-resistant colonies were selected and scored for antivirus activity. Positive cultures were expanded to be frozen; cultures of particular interest were cloned by limiting dilution (1 cell/well) to assure monoclonality. Clones were screened, and positive clones were expanded for freezing and production of ascitic fluid.

Production of ascitic fluid. Female BALB/c mice 10–12 wk old were injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane, Sigma Chemical Co.) 7 days before receiving an intraperitoneal injection of 2×10^6 antivirus antibody secreting hybridomas suspended in 1.0 ml of phosphate-buffered saline (PBS), pH 7.2 (13). Ascitic fluid developed 2–3 wk after cell injection, and fluid was collected every other day for a period of 1 wk by intraperitoneal paracentesis using a sterile, 16-gauge hypodermic needle. Ascitic fluid was centrifuged at 8,000 rpm for 10 min. The cellular pellet was discarded, and the supernate was treated with 5% sodium dextran sulfate and 11.1% calcium chloride (2,25) to remove lipoproteins. After centrifugation at 10,000 rpm for 10 min, supernate was saved, and immunoglobulin was precipitated by 50% ammonium sulfate. Centrifugation at 10,000 rpm generated pellets which were resuspended in 1× PBS, pH 7.2.

Assays. Culture fluids were tested for antibodies against BYDV-PAV-IL 27 and 33 days after fusion and for antibodies against BYDV-RPV-IL 15 and 17 days after fusion. Fluids were assayed by two types of ELISA on microtiter plates (Immulon I, Dynatech Laboratories, Inc., Chantilly, VA). All reagents were 100 μl/well, except blocking steps which were 200 μl/well. In the first assay, plates were coated directly with purified virus (400 ng/ml in carbonate buffer, pH 9.6) for 2 hr at 37 C and blocked with

TABLE 1. Reactions in triple antibody sandwich enzyme-linked immunosorbent assay of monoclonal antibodies produced against two Illinois barley yellow dwarf virus (BYDV) strains with isolates of the PAV serotype (group I)

BYDV isolate	Monoclonal antibody		
	PAV-IL-1	RPV-IL-1	RPV-IL-5
California	0.95[a]	0.01	0.02
Illinois	1.48	0.02	0.03
New York	1.21	0.01	0.03
North Dakota	1.01	0.02	0.05
Australia-Victoria	0.44	0.01	0.03
Canada-Y8801	1.74	0.03	0.06
Canada-Y8802	1.23	0.03	0.05
England	0.98	0.04	0.09
Ethiopia-W9a-88	0.52	0.01	0.04
Italy	0.97	0.03	0.07
Jordan-JW13-87	0.45	0	0
Jordan-JW43-87	0.61	0	0
Morocco-M60-87	1.14	0	0
South Africa-RSE	1.16	0.02	0.06
Syria-SB9-87	0.66	0	0.01
Syria-SB9-88	0.89	0.02	0.03
Tunisia-TB23-87	0.45	0	0.01
Uninoculated oats	0.07	0.03	0.08
Buffer	0	0	0

[a] Values are means of absorbances at A_{405} of triplicate wells after overnight incubation at 4 C. Samples with mean values greater than twice the mean of the negative control (uninoculated leaves of *Avena byzantina* 'Coast Black') are considered positive.

TABLE 2. Reactions in triple antibody sandwich enzyme-linked immunosorbent assay of monoclonal antibodies produced against two Illinois barley yellow dwarf virus (BYDV) strains with isolates of the RPV serotype (group II)

BYDV isolate	Monoclonal antibody		
	PAV-IL-1	RPV-IL-1	RPV-IL-5
California	0.03[a]	1.03	1.59
Illinois	0.04	1.08	1.46
New York	0.05	1.27	>2.00
Australia-Victoria	0	0.35	0.37
Canada-Comeau	0.06	1.08	1.76
Canada-Y6903	0.06	1.39	>2.00
England	0.13[b]	1.69	>2.00
Uninoculated oats	0.04	0.03	0.08
Buffer	0	0	0

[a] Values are means of absorbances at A_{405} of triplicate wells after overnight incubation at 4 C. Samples with mean values greater than twice the mean of the negative control (uninoculated leaves of *Avena byzantina* 'Coast Black') are considered positive.

[b] Uninoculated oat cultivar Avalanche, the host cultivar for the samples from England, had a mean absorbance of 0.09 in this PAV-IL-1 assay; therefore, the sample was rated negative.

TABLE 3. Isolates of barley yellow dwarf virus (BYDV) not detected in triple antibody sandwich enzyme-linked immunosorbent assay by monoclonal antibodies produced against two Illinois BYDV strains

Group I: MAV-like	Group I: SGV-like	Group II: RMV-like
California[a]	New York	New York
New York	Canada-Y7404	Australia-Queensland
Australia-Victoria	Canada-Y8516	Italy
Canada	Morocco-M49-87	
England		
Ethiopia-B9-88		

[a] No samples had mean values greater than twice the mean of the negative control (uninoculated leaves of *Avena byzantina* 'Coast Black'); therefore, none are considered positive.

4% polyvinyl pyrrolidone-40 (PVP-40) in PBS for 2 hr at 37 C. Between steps, plates were rinsed three times with PBS plus 0.05% Tween 20 (PBS-T) (4). Plates coated with carbonate buffer alone and blocked with PVP-40 were used as controls. In the second assay, a TAS-ELISA, plates were coated with virus-specific rabbit polyclonal immunoglobulin (Ig) (1 μg/ml) made against either BYDV-PAV-IL (5) or BYDV-RPV-IL (19). Virus from sap of leaves and roots (1:1) of infected oats (*Avena byzantina* C. Koch 'Coast Black') ground in PBS-T (1 g/3 ml) was trapped during overnight incubation at 4 C; extracts from uninoculated oats incubated on Ig-coated plates were used as controls. In both assays, culture fluids were incubated 1.5–2 hr at 37 C, and reacted monoclonal antibodies were detected with alkaline phosphatase-labeled goat anti-mouse antibodies (Hy-Clone Laboratories, Inc., Logan, UT) incubated overnight at 4 C, followed by *p*-nitrophenol phosphate substrate (Sigma Chemical Co.) (1 mg/ml in 10% diethanolamine) incubated for 1 hr at room temperature and then overnight at 4 C.

Nomenclature and characterization of monoclonal antibodies. Monoclonal antibodies were numbered with prefixes indicating the virus strain used for immunization (PAV or RPV) and the location in which they were made (Illinois)—for example, PAV-IL-1. Immunoglobulin subclasses were determined with a ZYMED Mono Ab-ID EIA kit A (ZYMED Laboratories, Inc., San Francisco, CA). The dilution end point for each monoclonal antibody was determined in two assays. All reagents were used at 100 μl/well, except blocking steps which were 200 μl/well. In TAS-ELISA, microtiter plates were coated with polyclonal Ig to trap virus from sap extracted from infected oat leaves; sap extracts from uninoculated leaves were used as controls. In the second assay, purified virus in carbonate buffer, pH 9.6 (50 ng/ml) was used to coat plates for 2 hr at 37 C. In both assays, plates were blocked after coating with PBS-T plus 0.1% nonfat dry milk (PBS-T+M) for 1 hr at room temperature (approximately 25 C).

Electron microscopy. Purified BYDV-PAV-IL was pelleted, and equal amounts were resuspended in either phosphate buffer, pH 7.0, or carbonate buffer, pH 9.6, and incubated at 37 C for 2 hr. Drops of each virus preparation were placed on Parafilm, and collodion, carbon-coated grids were floated on the drops for 1 min. Grids then were moved sequentially to 2 drops of water to rinse them and were stained with 2% uranyl acetate by holding them with forceps and running 8 drops of stain over each grid. After drying on filter paper, grids were viewed in a Hitachi 600 electron microscope (Hitachi Instruments, San Jose, CA) operating at 75 kV.

Use of monoclonal antibodies as coating antibody. Purified IgG (1 μg/ml) from each of the three ascitic fluids was coated on microtiter plates (Linbro, Flow Laboratories, Inc., McLean, VA) either for 1 hr at 37 C or overnight at 4 C. Plates were blocked with PBS-T+M as above. Sap extracted from uninoculated oat leaves and PAV-IL- or RPV-IL-infected oat leaves (1 g/3 ml PBS-T+M) was used for serial twofold dilutions which were added in duplicate wells and incubated overnight at 4 C. Polyclonal conjugates were diluted 1:1,000 in sap extract from uninoculated oats and incubated 2 hr at 37 C. Substrate (0.5 mg/ml) was incubated 1 hr at room temperature, then overnight at 4 C.

Detection of BYDV isolates. BYDV-infected and uninoculated tissues, dried for shipment according to Lister et al (16), were requested from BYD researchers around the world. Only isolates of previously determined serotype were included in this study. Thirty-seven isolates were included (Tables 1–3): 17 PAV-like, seven RPV-like, six MAV-like (transmitted specifically by *S. avenae*), four SGV-like (transmitted specifically by *Schizaphis graminum* Rond.) and three RMV-like (transmitted specifically by *R. maidis* Fitch) (10,22). Detection of isolates was tested in TAS-ELISA. Plates (Linbro) were coated with polyclonal Ig (1 μg/ml) homologous to the monoclonal antibody to be used for detection (1 hr, 37 C) and blocked with PBS-T+M as above. Samples were ground with mortars and pestles, first in liquid nitrogen, then in PBS-T+M (0.15 g/3 ml) and incubated in wells overnight at 4 C. Monoclonal antibodies purified from ascitic fluids were diluted 1:500 (RPV-IL-1 and RPV-IL-5) or 1:1,000 (PAV-IL-1) in PBS-T+M. Rabbit-anti-mouse (RAM) conjugate (Sigma Chemical Co.) was diluted 1:1,000 in PBS-T+M. Monoclonal antibodies and conjugate were each incubated for 2 hr at 37 C. Substrate (0.5 mg/ml) was incubated 1 hr at room temperature, then overnight at 4 C. Samples were considered positive when the mean absorbance of triplicate wells was greater than two times the mean absorbance of control wells (uninoculated tissue).

RESULTS

Production of monoclonal antibodies. When hybridoma culture lines were assayed for production of monoclonal antibodies against each virus strain, 66 of 192 (34.4%) were positive for BYDV-RPV-IL and 10 of 48 (20.8%) were positive for BYDV-PAV-IL. Two RPV lines and one PAV line that gave strong reactions with the homologous virus in infected sap were cloned, increased in ascitic fluid, and used in studies described herein. These monoclonal antibodies were designated PAV-IL-1, RPV-IL-1, and RPV-IL-5.

Characterization of monoclonal antibodies. Monoclonal antibody subclasses and titers determined with the homologous virus in sap extracts from infected oat tissue or with purified homologous virus coated on microtiter plates are summarized in Table 4. Suspension of BYDV-PAV-IL in the basic buffer used during the coating step of ELISA protocols (carbonate, pH 9.6) caused disruption of viral particles. This disruption could be visualized directly in the electron microscope (Fig. 1) and indirectly by the loss of a distinct ultraviolet-absorbing band in linear sucrose density gradients used to analyze treated virus preparations (data not shown). When each monoclonal antibody was tested for its ability to detect the homologous virus in plant sap, titers were 10^8 to 10^9 (Table 5). High nonspecific backgrounds with sap extracts from uninoculated oats were found when monoclonal antibodies were diluted only 10^{-2}. When the monoclonal antibodies were tested for ability to detect dissociated virus coated directly on microtiter plates for 2 hr at 37 C, titers dropped to 10^3.

Use of monoclonal antibodies as coating antibody. All three monoclonal antibodies were used successfully to trap homologous virus in double antibody sandwich (DAS)-ELISA. The highest absorbance values, obtained on plates with overnight coating and substrate incubation steps, were 1.8, 1.5, and 1.1 for PAV-IL-1, RPV-IL-1, and RPV-IL-5, respectively. Sap diluted 1:12 gave the highest absorbance values on PAV-IL-1-coated plates, whereas the most concentrated sap gave the highest values with the other two monoclonal antibodies. When plates were coated at 37 C for 1 hr, maximum absorbance values dropped on PAV-IL-1- and RPV-IL-5-coated plates to 0.8 but remained unchanged on RPV-IL-1-coated plates.

Detection of BYDV isolates. In TAS-ELISA, monoclonal antibody PAV-IL-1 detected all 17 isolates designated as PAV-like from four U.S. states and 10 other countries (Table 1). In TAS-ELISA, monoclonal antibodies RPV-IL-1 and RPV-IL-5 both detected all seven isolates designated as RPV-like (Table 2). The RPV-like strain from England had an unusually high

TABLE 4. Properties of monoclonal antibodies produced against two Illinois strains of barley yellow dwarf virus

Monoclonal antibody[a]	Isotype	Titer	
		Infected sap	Purified virus[b]
PAV-IL-1	IgG1, kappa	10^8	10^3
RPV-IL-1	IgG1, kappa	10^8	10^3
RPV-IL-5	IgG2a, kappa	10^9	10^3

[a] Monoclonal antibodies in ascitic fluid purified with sodium dextran sulfate and ammonium sulfate as described in the text.

[b] Virus (50 ng/ml) suspended in carbonate buffer, pH 9.6, and incubated 2 hr at 37 C.

mean absorbance in the PAV-IL-1 system. However, when its mean absorbance (0.13) was compared with the mean absorbance (0.09) of uninoculated oats of the same cultivar, *A. sativa* L. 'Avalanche,' the sample was rated negative for PAV. None of the three Illinois monoclonal antibodies was able to detect any of the heterologous BYDV isolates tested, which included MAV-like, RMV-like, and SGV-like isolates (Table 3).

DISCUSSION

Although monoclonal antibodies against North American strains of BYDV have been produced (8,14), none is currently widely available for use in large-scale projects such as breeding or epidemiological studies. We report production of monoclonal antibodies to strains of the two most commonly detected serotypes of BYDV in North America: PAV and RPV. The monoclonal antibodies are capable of detecting a wide range of isolates of these serotypes both from North America and from many other parts of the world.

When used at dilutions of at least 1,000-fold, all three monoclonal antibodies had excellent signal-to-noise ratios for detection of homologous virus in sap extracts. At dilutions of 100-fold, noise levels, that is, background in sap extracts from uninoculated plants, were unacceptably high for all three monoclonal antibodies. This same phenomenon has been noted in other luteovirus TAS-ELISA systems (17) and is probably due to nonspecific binding of IgG to the microtiter plates at high IgG concentrations. Compared with DAS-ELISA systems used in our laboratory for detection of BYDV strains, the TAS-ELISA systems reported here have threefold to fivefold higher signal-to-noise ratios. This improvement also has been reported for other BYD monoclonal antibody systems (21). Because the monoclonal antibodies were able to detect their homologous viruses at antibody dilutions several orders of magnitude greater than 10^3, they may be useful for detection of very low concentrations of virus in infected tissue.

None of the three BYDV monoclonal antibodies reacted well with virus that had been incubated at 37 C in the carbonate buffer routinely used for coating microtiter plates. There is strong evidence to indicate that BYDV strains, and probably other luteoviruses, dissociate under these conditions (8). For example, we have obtained similar results with BYDV-RPV-IL and with potato leafroll virus (D'Arcy and Martin, *unpublished data*). It is likely therefore that the BYDV monoclonal antibodies discussed herein react with epitopes that are conformational and are lost upon virion dissociation. The low level of reactivity remaining after the virions were incubated 2 hr at 37 C in carbonate buffer is probably due to reaction with particles that were incompletely dissociated. The dissociation of BYDV strains in carbonate buffer occurs quite rapidly during incubation at high temperatures and more slowly at low temperatures. For example, when RPV-IL-1 and RPV-IL-5 were titered against purified BYDV-RPV-IL (50 ng/ml) coated in carbonate buffer overnight at 4 C, titers were 10^7 for both clones (Murphy and D'Arcy, *unpublished data*). This suggests that, if plate-trapped virus is to be used in an ELISA to screen for monoclonal antibodies, the temperature at which coating is done will determine which monoclonal antibodies are detected. Screening with virus coated at 37 C was used in an attempt to detect monoclonal antibodies representing internal virus epitopes (cryptotopes). No such monoclonal antibodies were found.

All of the three monoclonal antibodies described herein were used successfully to trap homologous virus in DAS-ELISA, most efficiently when plates were coated overnight at 4 C. The apparent inhibition of antigen binding by PAV-IL-1-coated antibody at high sap concentrations is similar to that found for a U.K. monoclonal antibody (MAC92) to an RPV-like isolate of BYDV (L. Torrance, *personal communication*). MAC92 is more efficient as a detecting antibody when sap is diluted 10- to 40-fold. Optimal sap concentrations must be determined for each monoclonal antibody used as a coating reagent.

Each of the monoclonal antibodies discussed herein has strong cross-reactions with other luteoviruses (6; Murphy and D'Arcy, *unpublished data*). PAV-IL-1 cross reacts with potato leafroll virus; RPV-IL-1 cross reacts with bean leafroll virus; and both RPV monoclonal antibodies cross react with beet western yellows virus. None of these cross-reactions will limit the usefulness of the monoclonal antibodies for BYDV detection and diagnosis in grass species because all the other luteoviruses detected infect dicotyledonous species.

The use of monoclonal antibodies for detection and diagnosis of luteoviruses has several advantages over polyclonal antisera. Hybridoma cell lines provide a continuous supply of a defined reagent. The clones described herein, which had been stored at −80 C for almost 2 yr, recently have been regrown, and the specificity and sensitivity of virus detection remain unimpaired. A second advantage is the specificity of the reagents. Monoclonal antibodies clearly differentiate among strains of BYDV within groups I and II, where polyclonal antisera may not (21,23). Of course, a panel of appropriate monoclonal antibodies will be required for detection and diagnosis of all strains of BYDV present in a location. There also is the possibility that monoclonal antibodies may be so specific that they fail to detect some isolates of the virus that lack the epitope to which they were made. However, if a monoclonal antibody has been demonstrated to recognize many known strains of the virus, as have the BYDV monoclonal antibodies discussed herein, that possibility becomes more remote.

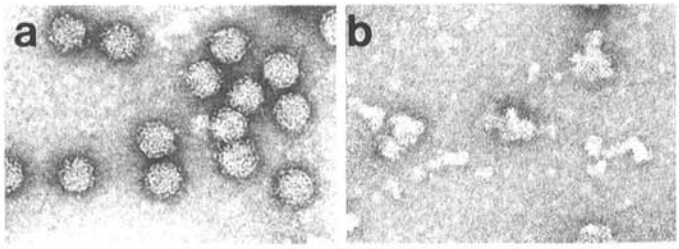

Fig. 1. Electron micrographs of purified barley yellow dwarf virus. PAV-IL strain incubated in **A,** phosphate buffer, pH 7.0, or **B,** carbonate buffer, pH 9.6, for 2 hr at 37 C and stained with 2% uranyl acetate. ×150,000.

TABLE 5. Reactions in triple antibody sandwich enzyme-linked immunosorbent assay of monoclonal antibodies produced against two Illinois barley yellow dwarf virus strains with extracts from leaves of oats infected with the homologous virus strain or from uninoculated oats

Antibody dilution	PAV-IL-1		RPV-IL-1		RPV-IL-5	
	Infected[a]	Control	Infected	Control	Infected	Control
10^{-2}	>2.00[b]	1.51	0.64	0.30	0.97	0.62
10^{-3}	1.44	0.09	0.61	0.02	0.87	0.05
10^{-4}	1.10	0.01	0.69	0	1.11	0.01
10^{-5}	0.67	0	0.65	0	1.14	0
10^{-6}	0.22	0	0.49	0	0.91	0
10^{-7}	0.05	0	0.19	0	0.47	0
10^{-8}	0.01	0	0.03	0	0.13	0
10^{-9}	0	0	0	0	0.02	0
10^{-10}	0	0	0	0	0	0
Buffer	0	0	0	0	0	0

[a]Extracts were 1 g tissue/3 ml of phosphate-buffered saline-Tween plus 0.1% nonfat dried milk.
[b]Values are means of absorbances at A_{405} of duplicate wells for each sample after overnight incubation at 4 C.

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