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MONOCLONAL ANTIBODIES AGAINST POTATO VIRUS A – – COMPETETIVE BINDING TESTS

N. ČEŘOVSKÁ, M. FILIGAROVÁ

Institute of Experimental Botany Czechoslovak Academy of Sciences, Na Karlovce 1a, 160 00 Prague 6, Czechoslovakia

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Summary. – Six mouse monoclonal antibodies (MoAbs) against potato virus A (PVA) were tested. One of them (PVA 534) reacted only with complete virions and was apparently specific for epitopes dependent on quaternary structure. MoAb PVA 328 recognized the virus antigen only after its dissociation into subunits. MoAb PVA 328 must have reacted with a cryptotope of the antigen. MoAb PVA 151 and 290 appeared to be conformation independent and reacted with exposed regions on native virus particles as well as on the surface of dissociated coat protein subunits. Two other MoAbs (PVA 187 and 634) did not recognize subunits or the virions adsorbed directly to the microtiter plate. This seems to be an evidence of metatope existence. The results of competetive binding tests combined with the reaction patterns of individual MoAbs to different potyviruses indicate that the MoAbs are specific for 6 distinct epitopes.

Key words: potato virus A; monoclonal antibodies; competetive binding tests

Introduction

PVA belongs to potyvirus group (Brandes and Wetter, 1959), which are elongated filamentous particles 720–790 nm long. PVA causes a widespread and economically damaging disease in potatoes. Therefore a large number of potato samples are tested by ELISA for quarantine and certification purposes for its presence. Standardization and production of high quality immunoreagents is readily achieved with MoAbs, which have also been used succesfully for the antigenic analysis of various plant viruses (Altschuh *et al.,* 1985; Koenig and Torrance, 1986; Sober *et al.,* 1988). Dore *et al.* (1988) found three groups of antibodies recognizing virus (TMV) or viral protein in two types of ELISA, which use antibody-coated or antigen-coated plates, respectively. From the reactivity of these MoAbs three types of epitopes could be distinguished (Van Regenmortel, 1982). The first group of epitopes arising from the quaternary

structure of the virus is called neotopes, the second group is cryptotopes, i.e. epitopes present in the isolated protein but absent in the assembled viral particle. The third group of MoAbs recognizes both virus and viral protein in ELISA with antibody coated plates. The epitopes are called metatopes. Using mouse MoAbs to PVA Boonekamp *et al.* (1990) found four different antigenic determinants. Since epitope 1 is found only in purified PVA, it seems to arise during purification of the virus. The epitopes recognized by MoAbs 3, 4 and 5 reacted with both purified and non-purified PVA. In this paper we describe the antigenic properties of six mouse MoAbs against PVA-LI (isolate from potato cv. Lichte Industrie) by various types of ELISA and competetive binding tests.

Materials and Methods

Viruses. The isolate of PVA by the Institute of Potatoes Research and Breeding, Havlíčkův Brod, Czechoslovakia, was grown in *Nicotiana tabaccum* cv. Samsun. The leaves of infected plants were harvested 3–6 weeks after inoculation. The virus was purified by high speed centrifugation followed by equilibrium density gradient centrifugation in cesium chloride according to Čeřovská *et al.* (1991). Virus concentration was estimated spectrophotometrically ($A_{260}^{0.1\%} = 2.8$; Stace-Smith and Tremaine, 1970). The purified virus was used for rabbit anti-PVA antiserum production and also for the production of mouse MoAbs. Dissociated PVA coat protein (PVAP) was prepared according to Francki and McLean (1968). The subunit antigen was prepared by freezing purified virus in 3 mol/l LiCl for at least 16 hr at -20 °C. After thawing, intact virus particles and RNA were removed by centrifugation at 160 000 x g for 90 min and the soluble coat protein subunits were diluted in an appropriate buffer. The solution was used immediately or stored at -20 °C.

MoAbs were prepared in collaboration with Dr. F. Franěk from the Institute of Molecular Genetics of Czechoslovak Academy of Sciences, Prague. The hybridomas were generated by fusion of spleen cells from immune mice with the mouse myeloma cells Sp2/0 - AG14 (Galfre and Milstein, 1981). The hybrid population in HAT medium was distributed into 800 wells of microtiter plates. Supernatants from growing cultures were tested for the presence of PVA specific antibodies by ELISA-1. The cultures secreting specific MoAbs were cloned by limiting dilution and than propagated to obtain inocula for the production of ascites in BALB/c mice primed with mineral oil.

Preparation of immunoglobulins (Ig) and conjugation with enzyme. IgG was precipitated from mouse ascitic fluid or from rabbit antiserum with 50 % (v/v) saturated $(NH_4)_2SO_4$ solution and adjusted to pH 7.4 with 1 mol/l NaOH. The precipitate was dissolved in, and dialyzed against PBS. Samples of IgG were conjugated to alkaline phosphatase (Boehringer) by the glutaraldehyde method described by Avrameas (1969), using 2 500 units of enzyme/mg globulin.

ELISA. Extracts of PVA-infected or virus-free tobacco or potatoes, or the purificate of PVA were used as an antigen. For some assays, other viruses belonging to potyvirus group were used (potato virus Y (PVY), plum pox virus (PPV), turnip mosaic virus (TuMV), tulip breaking virus (TBV), fresia mosaic virus (FMV), bean yellow mosaic virus (BYMV) and iris severe mosaic virus (ISMV)).

ELISA-1 (triple antibody sandwich ELISA). The wells of microtiter plates (Institute of Molecular Genetics of Czechoslovak Academy of Sciences, Prague) were incubated for 3 hr at 37 °C after being coated with rabbit anti-PVA or polyclonal antibodies against PVY and PPV, or anti-TuMV (supplier Dr. Špak, Institute of Molecular Biology of Plants of Czechoslovak Academy of Sciences, České Budějovice), anti-TBV_{RE}, -TBV_{RD}, -ISMV, -BYMV, -FMV (supplier Dr. A.F.L.M. Derks, Bulb Research Station, Lisse, Netherlands) at 1 μg/ml (for PVAP at 10 μg/ml) in 50 mmol/l sodium carbonate buffer (coating buffer) at pH 9.6 (200 μl/well). After washing the wells four times with PBS containing 0.05 % Tween-20 (PBST), the samples (Table 1) in PBST + 0.2 % BSA + 2 % polyvinyl-pyrrolidone, M_r 20 000 (PBST + PP) were added and incubated overnight at 4 °C. The wells were washed as above and isolated MoAbs (1 μg/ml) were incubated for 2 hr at 37 °C. After washing

a swine anti-mouse IgG-alkaline phosphatase conjugate (SWAM-AP, Sevac, Czechoslovakia) in a 1:1000 dilution, the conjugate was incubated for 4 hr at 37 °C and then detected with p-nitrophenyl phosphate in 1 mol/l diethanolamine buffer, pH 9.8.

ELISA-2. For the plate-trapped antigen form of indirect ELISA (Mowat, 1985) three steps were performed, which were separated by washing the wells as described previously. In step 1 the wells contained purified virus or viral coat protein in a coating buffer, in step 2 this was replaced by MoAbs (5 μg/ml) in PBST + PP buffer and in step 3 the linked MoAbs were detected using SWAM-AP in dilution 1:1000.

ELISA-3 (double antibody sandwich ELISA). This type of ELISA was performed according to Clark and Adams (1977) by using MoAbs as coating antibody and alkaline phosphatase labelled MoAbs (MoAbs-AP) as a second antibody. In all types of ELISA tests the A_{405} was measured with the ELISA Dynatech Minireader II.

Competetive binding tests. Unconjugated MoAbs were tested individually for their ability to block the subsequent binding each of the set of MoAbs conjugated to alkaline phosphatase. Wells of microtiter plates were coated with anti-PVA IgG (1 μg/ml, 37 °C, 4 hr), washed, pure virus antigen in PBST + PP was added and incubated overnight. Unconjugated blocking MoAb in PBS + PP was then added to each well at a concentration found in preliminary experiments to completly inhibit the subsequent binding of the homologous MoAb conjugate with each MoAb. After incubation for 4 hr at 37 °C and without washing the plates, 10 μl MoAb conjugate was added to each well to give a final conjugate concentration as shown in Table 4. After an additional overnight incubation, the plates were washed and substrate added as described above. The incubation times with substrate varied to compensate for the different rates at which the substrate was hydrolyzed by different conjugates. The proportion by which A_{405} was decreased when each MoAb was used to block the binding of each conjugate was calculated. If this proportion exceeded 50 %, the antigenic sites were considered to be topographically related.

Results and Discussion

Differentiation of PVA epitopes

The epitope specificity of the different MoAbs was determined by ELISA with the PVA or PVA capsid protein adsorbed to plates (ELISA-2) or by indirect type of ELISA (ELISA-1). MoAbs to PVA have been previously produced (Boonekamp *et al.*, 1990) and some of them were shown to recognize epitopes of both

Table 1. Source of virus isolates used

Virus	Leaf tissue tested
PPV_1	*Nicotiana glutinosa* x clevelandii
PVV_2	*Nicotiana glutinosa* x clevelandii
PPV_3	*Nicotiana glutinosa* x clevelandii
PVY^0	*Nicotiana tabaccum*
PVY^N	*Nicotiana tabaccum*
TuMV	cabbage
TBV_{RE}	tulip, bulb
TBR_{RD}	lily, bulb
FMV	fresia, flower
BYMV	fresia, flower
ISMV	canna
PVA_{LI}	*Nicotiana tabaccum*

Table 2. Reactivity of MoAbs in different ELISA procedures with virions and viral coat protein

MoAbs	ELISA-1		ELISA-2	
	a	b	a	b
PVA 151	+ *	+	+	+
PVA 290	+	+	+	+
PVA 328	-	+	+	+
PVA 187	+	+	-	-
PVA 634	+	+	-	-
PVA 534	+	-	-	-

a - reaction with virions
b - reacttion with viral coat protein
ELISA-1 - wells were coated with rabbit anti-PVA IgG, antigen was detected with specific MoAb, which in turn was detected with swine anti-mouse IgG-alkaline phosphatase conjugate; ELISA-2 - plate-trapped antigen form of indirect ELISA.

* A_{405} values higher than three times background (+) and in the range of background (-).

intact virus particles and dissociated coat protein subunits. Analysis of the six MoAbs (Table 2) also provides evidence for the presence of cryptotopes, neotopes and metatopes associated with PVA particles. It is known that upon binding to plastic in the plate-trapped antigen form of indirect ELISA, virus proteins expose epitopes other than those detected by ELISA-1 (Al-Moudallal *et al.*, 1984; Tremaine *et al.*, 1985). An antigen trapped indirect ELISA type assay on EM grids showed that if 10 % of the virions had been degraded, only subunits and no intact TMV particles became attached to the plastic (Dore *et al.*, 1988). During our studies aimed at characterizing the binding specificity of these MoAbs it was found that three of them (PVA 151, 290, 328) reacted with subunits of the viral coat protein. These results were obtained when the antibodies were tested by an ELISA-2b in which the microtiter wells had been coated directly with PVAP. In subsequent experiments it was shown that when the more sensitive ELISA-1b was used in which the wells were first coated with viral antibody, two other MoAbs (PVA 187, 634) were able to bind to PVA protein subunits, while one reacted only with complete virions (PVA 534). The results presented in Table 2 show that in the ELISA-1a procedure reacted all MoAbs with virions (expect PVA 328), whereas in the ELISA-1b only PVA 534 did not react with subunits of viral protein. Presumbly MoAb PVA 534, which reacts only with the virus, is directed against epitopes specific for the quaternary structure of virions, i.e. against the so called viral neotopes (Van Regenmortel, 1982). This neotope specific antibody did not react with the virions in the ELISA-2a procedure. It seems that when the virus is directly adsorbed to the plastic surface the neotope conformation is disrupted and the virions become antigenically similar to subunits. Some of the MoAbs (PVA 151, 290) bound to

Table 3. Reaction of MoAbs to PVA with different potyviruses in ELISA-1

Virus	MoAbs					
	PVA 151	PVA 187	PVA 290	PVA 328	PVA 534	PVA 634
PVA_{LI}	+++	+++	+++	+++	+++	+++
PPV_1	0	0	0	0	0	0
PPV_2	0	0	0	0	0	0
PPV_3	0	0	0	0	0	0
TuMV	+	++	0	+	0	++
TBV_{RE}	0	0	0	0	0	+
TBV_{RD}	0	0	0	0	0	0
FMV	+	+	0	0	+	0
BYMV	0	0	+	+	0	0
ISMV	++	+++	+++	+	++	+++

+++ A_{405} = 0.6 - 2.5 (1 hr after adding substrate)
++ A_{405} = 0.1 - 0.6 (1 hr after adding substrate)
+ A_{405} = 0.1 (overnight)
0 A_{405} = 0.02 (overnight)

As unrelated MoAb the anti-PLRV MoAb was used. The A_{405} values were 0.02 - 0.05.

subunits in the ELISA-2a, b procedure and also reacted with virions in both types of ELISA. MoAbs PVA 151 and PVA 290 appear to be conformation-independent as they reacted with regions exposed on native particles as well as on the surface dissociated coat protein subunits. Secondly, MoAb PVA 328 must have reacted with a cryptotope because it recognized the virus antigen only after dissociated of particles into subunits. It bound the viral coat protein subunits in both ELISA types (Table 2), but it did not react with the intact virus particle. Other two MoAbs (PVA 187, 634) did not recognize subunits or virions bound directly to the microtiter plate surface in the ELISA-2 procedure but reacted with both of them during the ELISA-1 procedure. These results provided evidence for the existence of metatopes.

Epitope mapping

The MoAbs differed markedly in their ability to react with other potyvirus isolates in indirect ELISA-1 (Table 3). The different patterns of MoAbs reactivity indicate that each of the six MoAbs detect different epitope. To obtain further information on epitope discrimination and topological relationship, a series of competetive binding tests was done in which the ability of six MoAbs to block the subsequent reaction of antibody-trapped PVA particles with six MoAbs conjugates was assessed. The results of these blocking ELISA and ELISA-3 (Table 5) indicate, that the blocking MoAbs, and therefore the epitopes, can be divided into several groups (Table 4).

Table 4. Competitive binding tests with MoAbs to PVA

Blocking MoAbs	Conjugate MoAbs					
	PVA 151	PVA 187	PVA 290	PVA 328	PVA 534	PVA 634
PVA 151	+++	0	+++	++	0	+
PVA 187	+	+++	0	+	0	0
PVA 290	+++	0	+++	++	0	+
PVA 328	+++	+	+	+++	0	++
PVA 534	++	0	0	++	+++	0
PVA 634	0	+	++	+	++	+++

A_{405} was recorded after incubation at 37 °C after 60 min. or following additional 16 hr at 4 °C. The proportions by which A_{405} was decreased when the blocking MoAb was used were: 0 (< 15 %), + (15–50 %), ++ (51–80 %), +++ (> 80 %).

MoAb PVA 187. This MoAb does not block the reaction with other PVA MoAbs, e.g. its epitope is topologically different from MoAbs PVA 151, PVA 290 and PVA 328. Binding of MoAb PVA 151 to the native virus (blocking ELISA) prevented the binding of PVA 290, 328 and vice versa, suggesting that these three epitopes are topologically related. The capture of PVA by MoAb PVA 151 followed by labelled MoAb PVA 151 (Table 5) gives a positive signal, indicating that captured MoAb is unable to saturate the PVA epitopes on the opposite side of the virus. The capture of PVA by MoAb PVA 290 followed by labelled PVA 151 resulted in lower absorbance in ELISA. It depends on the capacity of this MoAb to bind the virus (data not shown).

Table 5. Reactivity of MoAbs in ELISA-3

MoAbs	MoAbs-AP					
	PVA 151	PVA 187	PVA 290	PVA 328	PVA 534	PVA 634
PVA 151	> 2.45	0.41	0.52	0.79	0.46	0.00
PVA 187	1.73	0.15	0.00	0.23	0.70	0.00
PVA 290	0.80	0.16	0.39	0.30	0.54	0.00
PVA 328	> 2.45	0.18	0.26	0.45	0.23	0.14
PVA 534	0.91	0.22	0.37	0.49	0.57	2.09
PVA 634	0.00	0.00	0.42	0.25	0.00	0.00

ELISA-3: MoAbs (1 μg/ml), MoAbs-AP (1:1000)
The background A_{405} values (PBS + PP buffer) were < 0.01.

MoAb PVA 534. Binding MoAb PVA 534 to native virus in blocking ELISA did not block MoAbs PVA 187, 290 and 634. This indicates no conformational changes of epitopes of these MoAbs by binding PVA 534 and special separation of these epitopes (topologically different epitopes) from PVA 534. The results in Table 4 also indicate a lack of reciprocity among some of these MoAbs in competition for binding these MoAbs. PVA 151 blocks the binding of PVA 187 completely, but PVA 187 blocks the binding of PVA 151 by only about 20 %. A possible explanation of these results is that the epitopes detected by the second MoAb are more restricted in the population of virus particles than the first one. The results (Table 4) indicate that some of the MoAbs reacting with epitopes are topologically related. This interpretation is in line with the evidence that discrete epitopes occur close together so that they behave as one site in competitive binding tests (Schmaljohn *et al.*, 1983). All presented results (Tables 3, 4 and 5) provided evidence that our MoAbs to PVA were specific for six different epitopes.

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