Acta virol. **37:** 61–67, 1993

CONSTRUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO ALFALFA MOSAIC VIRUS

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Received April 10, 1992; revised July 1, 1992

Summary. – Seven monoclonal antibodies (MoAbs) to alfalfa mosaic virus (AMV) isolate T6 were prepared and characterized. One of them (*amv* 4) reacted with the cryptotope, another (*amv* 5) with the neotope and the remaining five with various metatopes. Five MoAbs derived from mouse ascitic fluid reacted in immunodiffusion test with native virus and two MoAbs with AMV coat protein. In competetive ELISA, homologous MoAbs caused 80–100 % inhibition of the reaction, but some reactions were also affected by heterologous MoAbs. The MoAbs raised against AMV isolate T6 could not by used for differentiation of the four AMV pathotypes examined.

Key words: alfalfa mosaic virus; monoclonal antibodies; ELISA; immunodiffusion

Introduction

AMV is very common in Czechoslovakia. It occurs in several pathotypes (Musil *et al.*, 1966), but no differences between them could be established in several serological reactions with polyclonal antisera. Similarly in the immunodiffusion test, Gallo (1977) found no differences between the so-called necrotic and chlorotic AMV strains of different pathogenicity. MoAbs are capable of revealing even minute differences between biologically distinct AMV isolates (Hajimorad and Francki, 1988; Hajimorad *et al.*, 1990). The AMV coat protein consists of several epitopes of different structure and arrangement (Halk *et al.*, 1984; Dekker *et al.*, 1989; Hajimorad *et al.*, 1990). The aim of the present study was to produce MoAbs to AMV isolate T6 (Gallo, 1980) and to characterize them.

Materials and Methods

Antigens. AMV isolates T6 (Gallo, 1980), TrM6A, TpMl and MSM (Musil *et al.*, 1966) were propagated in pea (*Pisum sativum* cv. Juran) plants and purified as described by Gallo (1980). Purified

virus suspensions in 0.01 mol/l phosphate buffer pH 7.00 were stored at -80 °C. Preparation of virus coat protein was obtained by disrupting the virus in disruption buffer (Wu and Bruening, 1971) at 100 °C for 5 min or at 65 °C for 30 min. Viral antigens were diluted in buffers required by the given ELISA techniques. The same buffers were used to obtain a 10-fold dilution of sap from healthy pea plants included in the tests as control.

Construction of hybridomas. Three months old BALB/c mice (20 g) were immunized intraperitoneally on the days 0, 30 and 80 with 100 μg doses each of purified AMV T6 suspension mixed with an equal volume of complete Freund's adjuvant (Difco). An additional dose of 200 μg virus in phosphate buffered saline was given intravenously on the day 102. Three days latter blood samples were taken from the retroorbital sinus and assayed by ELISA for virus-specific antibody. The mice with highest antibody titer was killed and its spleen was dissected aseptically. The spleen cells were fused with SP2/0 mouse myeloma cells in the ratio of 4:1 by a modified method of Lane *et al.* (1986). Myeloma and hybrid cells were grown in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in an atmosphere containing 7.5 % CO_2. The medium contained 0.6 % $NaHCO_3$ and was supplemented with 0.005 % gentamycine, 1 mmol/l sodium pyruvate and 10 % heated horse serum. The selection medium (DMEM-HAT) used for cultivation of cells after fusion was supplemented with 0.1 mmol/l hypoxanthine, 0.16 mmol/l thymidine, 0.004 mmol/l aminopterine, 0.0028 mmol/l 2-mercaptoethanol, 2 % human umbilicord serum and 15 % horse serum. The cells were seeded in DMEM-2 × HAT mixed with an equal volume of conditioned medium LSP2/0 culture supernatants at a density of 3×10^5 cells per well of microtitration plate (Flow Laboratories). From 7 to 10 days after fusion there followed acreening by PTA-ELISA for hybridoma populations producing anti-AMV antibody. Selected hybridoma populations were subjected to 5-fold cloning (Torrance *et al.*, 1986). Mouse ascitic fluids were prepared according to Jones *et al.* (1990).

Isotyping of the MoAbs obtained was carried out by immunodiffusion test with commercial antisera against mouse immunoglobulin subclasses (Miles Laboratories).

Immunoglobulins from ascitic fluids were puriffied by ammonium sulphate precipitation and subsequent affinity chromatography on DEAE-Affi-gel blue (Bio-Rad) or DEAE 52-cellulose (Serva). Immunoglobulin concentrations were assayed spectrophotometrically (Gallo and Valenta, 1985).

ELISA. Four types of this assay were employed using buffers described by Clark and Adams (1977) without bacteriostatics and 100 μl total volume throughout. Plate-traped antigen ELISA (PTA-ELISA): direct binding of the antigen (1 μg/ml native virus or coat protein) proceeded in coating buffer at 4 °C for 18 hr. After washing, either media from hybridoma cultures or ascitic fluids dilluted in sample buffer from 1:100 to 1:4 096 000 were added and allowed to react at 35 °C for 1 hr on shaker incubator (Dynatech). Then there followed washing and addition of commercial peroxidase-labelled swine anti-mouse IgG (Institute of Sera and Vaccines, Prague) dilluted 1:5 000 in sample buffer supplemented with 5 % skim milk powder. The reaction proceeded at 35 °C for 1 hr and at 4 °C for 1 hr. Antibody-trapped ELISA (ATA-ELISA): the diagnostic plates were coated at 35 °C for 4 hr with rabbit anti-AMV IgG (polyclonal antiserum produced in our laboratory) dilluted in coating buffer to a 1 μg/ml concentration. Then there followed washing, addition of antigen and further steps as in PTA-ELISA. Competitive binding test ELISA (CBT-ELISA): the diagnostic plates were coated with anti-AMV IgG and allowed to react with the antigens as described under ATA-ELISA. After washing, non-labelled MoAbs were added in dillutions yielding an approximately equal absorbancy in PTA-ELISA. The reaction proceeded at 35 ° 5 for 3 hr on a shaker incubator. After washing, various dillutions (1:5 000 - 1:20 000) of alkaline phosphatase-labelled MoAbs in both homologous and heterologous combinations were added (Fig. 3). The reactions proceeded at 35 °C for 3 hr on a shaker-incubator. The results were compared with those of double antibody sandwich ELISA (Clark and Adams, 1977). The MoAbs were labelled with alkaline phosphatase Type VII S (Sigma) according to Gallo and Valenta (1985). Each combination was tested at least in duplicates and the tests were run twice.

The substrates for horse radish peroxidase and alkaline phosphatase were o-phenyle diamine (Fluka; 0.5 mg/ml) and 4-nitrophenyl phosphate bis (2-amino-2-ethyl-1,3-propanediol) salt (Serva; 0.8 mg/ml), respectively. The A_{490} and A_{410} values were read on a Dynatech spectrophotometer.

In all tests described above we used MoAbs produced in our laboratory against potato leafroll (Luteovirus) and broad bean stain (Comovirus) viruses as controls.

Double immunodiffusion (ID) tests were carried out in 0.8 % agarose (Serva), supplemented in some experiments with 2 % polyethylene glycol (PEG) 6 000 (Goding, 1986; Masalski and Harison, 1987), in 0.1 mol/l phosphate buffer pH 7.00. The precipitation bands were stained with amino black 10B (Serva).

Results

We obtained a total of 48 hybridomas producing anti-AMV MoAbs. Seven of these were subjected to cloning and all were found to be of the IgG class: five belonged to the IgG1 and two to the IgG2a subclass. In two types of ELISA (PTA, ATA), most of these MoAbs reacted with both native virus and coat protein in different titers. The differences in MoAb avidities to native virus and coat protein were more marked in ATA than PTA ELISA. MoAb *amv* 5 reacted only with native virus and MoAb *amv* 4 did so only with coat protein. We thus obtained MoAbs to three types of epitope, namely *amv* 4 to cryptotope, *amv* 5 to the neotope and the other MoAbs to the metatopes. For their characteristics see Table 1.

In ID tests, MoAbs derived from serum-free media reacted well with commercial antisera used for immunoglobulin isotyping, but not with AMV. By contrast, MoAbs from mouse ascitic fluids reacted in ID test as follows: with the exception of *amv* 4 and *amv* 18 all gave a precipitation reaction with native virus; only *amv* 4 and *amv* 10 reacted with virus coat protein (Table 1). Rabbit anti-AMV serum (Fig. 1A) obtained by intravenous immunization with native virus contained mainly antibody to native virus (in the form of purified virus or infected plant sap), as well as antibody to healthy pea proteins; no reaction was obtained with virus coat protein. MoAb *amv* 4 reacted only with virus coat

Table 1. Reaction of different MoAbs with AMV antigens in PTA ELISA, ATA ELISA and ID test

MoAb	Isotype	PTA ELISA*		ATA ELISA*		ID test**	
		NV	CP	NV	CP	NV	CP
amv 3	IgG1	64	2	1024	16	+	-
amv 4	IgG2a	32	1024	0.1	512	-	+
amv 5	IgG1	4	0.5	512	0.5	+	-
amv 10	IgG1	128	256	1024	512	+	+
amv 11	IgG1	64	1	512	32	+	-
amv 18	IgG2a	32	1	128	64	-	-
amv 20	IgG1	16	2	64	64	+	-

* Titer of MoAb (in thousands)
** Presence (+) or absence (-) of precipitin lines
NV - native AMV
CP - AMV coat protein

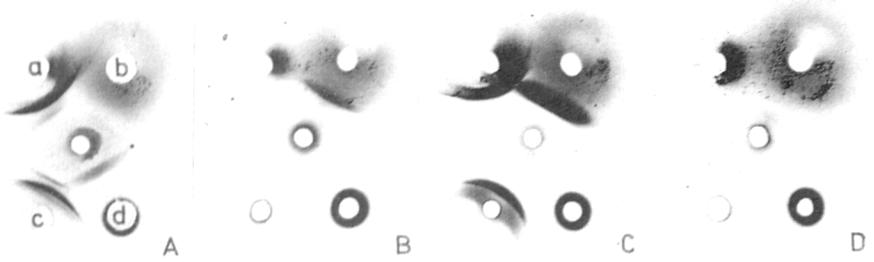

Fig. 1
ID tests of different MoAbs with different antigens
Antibodies: rabbit anti-AMVserum (A), MoAb *amv* 4 (B), MoAb *amv* 10 (C) and *plrv* (D). Native AMV (a), AMV coat protein (b), sap from AMV infected peas (c) and sap from healthy peas (d).

protein (Fig. 1B) and MoAb *amv* 10 gave a precipitation reaction with both native virus and coat protein (Fig. 1C). MoAb to PLRV used as control gave no ID reaction with any of the test antigens (Fig. 1D). The virus-specific MoAb gave no reaction with healthy pea proteins; we obtained such reactions when screening our hybridomas, but we did not examine the respective antibodies in detail. MoAbs *amv* 3, *amv* 5, *amv* 11 and *amv* 20 reacted in ID tests only with native virus (Fig. 2a), with the formation of a coalescing precipitin lines. Similarly, a coalescing precipitin line was produced by MoAbs *amv* 4 and *amv* 10 with coat protein, while the other MoAbs did not react (Fig. 2b). The presence of PEG in agarose had no effect on the formation of precipitin lines.

CBT ELISA showed that our MoAbs were directed against 7 different epitopes. An inhibition of the antigen-labelled antibody reaction higher than

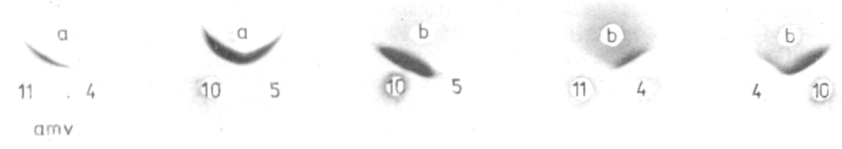

Fig. 2
ID tests of different MoAbs with native AMV and AMV coat protein
MoAbs: *amv* 4, 5, 10 and 11. Native AMV (a), AMV coat protein (b).

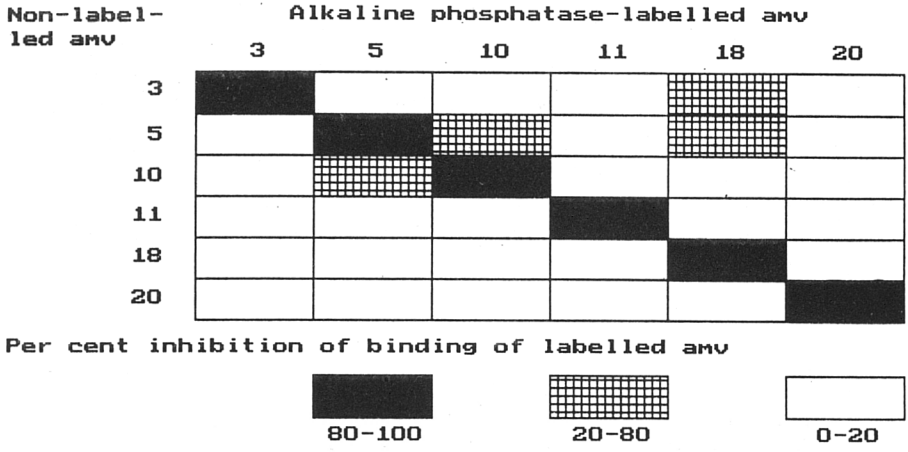

Fig. 3
Reaction of different MoAbs in CBT ELISA

80 % was obtained with all MoAbs in homologous reactions. With the exception of the homologous reaction, MoAbs *amv* 11, *amv* 18 and *amv* 20 did not inhibit any reaction of antigen with labelled MoAb. An inhibition in the range from 20 to 80 % was caused by MoAb *amv* 3 and *amv* 5 with labelled *amv* 18, and mutually by *amv* 5 and *amv* 10. The inhibition per cent varied greatly between individual experiments in spite of a standard procedure employed (Fig. 3). MoAb *amv* 4 was excluded from this testing, because in pilot experiments it caused no inhibition of the reaction of labelled MoAb with native virus.

ATA ELISA with the 7 MoAbs described above revealed no significant differences between the AMV pathotypes TrM6A, TpM1 and MSM used either as native virus or coat protein.

Discussion

Halk *et al.* (1984) and Halk (1986) were the first to prepare MoAbs to AMV; they found that in the course of immunization antibodies are formed which are capable to react with corresponding epitopes on native virus (neo- and metatopes) and with viral coat protein (cryptotopes). Similar conclusions were reached by Hajimorad *et al.* (1990) who moreover detected in AMV the presence of an epitope reacting with the respective MoAb only if the virus was fixed with glutaraldehyde. This epitope was presented in all AMV isolates they had examined. Hajimorad *et al.* (1990) raised their MoAbs by immunization with

a mixture of several AMV isolates (pathotypes) and found that the MoAbs were pathotype-specific. We used only one AMV pathotype for immunization and tested the resulting MoAbs with other AMV pathotypes which were at our disposal. In contrary to Hajimorad *et al.* (1990) we failed to detect any pathotype-specificity in our MoAbs what might be due to the different AMV pathotypes tested. This might suggest a great antigenic homogenity of the latter in spite of differences in pathogenecity (different host range, disease symptoms and replication dynamics).

We obtained two types of MoAbs isotype (IgG1 and IgG2a). Hajimorad *et al.* (1990) described IgG1 and IgM anti-AMV MoAbs. It is difficult to explain why we did not obtain any IgM MoAb. We found no relationship between MoAb isotype and the respective epitope: IgG1 detected the neotope (AMV 5) and metatopes (*amv* 3, 10, 11, 20), IgG2a detected the cryptotope (AMV 4) and metatope (*amv* 18).

In titrating MoAbs by two types of ELISA with two antigens (native virus and coat protein) we found that a MoAb reacting with the cryptotope (*amv* 4) reacted in PTA ELISA also with native virus, but it did not so in ATA ELISA. As distinct from other MoAbs, a MoAb to the neotope (*amv* 5) showed a low titer in PTA ELISA, and gave no or very slight reaction in ATA ELISA with coat protein. As revealed by CBT ELISA, the remaining MoAbs detected metatopes in a different manner. MoAb *amv* 10 reacted well both native virus and coat protein in PTA ELISA and ATA ELISA, as well as in ID test. A similar MoAb was described by Hajimorad *et al.* (1990).

The decresed ability of labelled MoAb *amv* 18 to react with homologous epitope after its reaction with MoAb *amv* 3 and *amv* 5 suggests that the reaction of the latter two MoAbs with their respective epitopes induced such conformational change in the epitope reacting with *amv* 18 that its reaction was partially inhibited. Reaction of *amv* 18 with its own epitope, however, did not inhibit reaction of labelled MoAbs *amv* 3 and *amv* 5 with their respective epitopes. In this case metatope reactions were involved. Similar inhibitions occured also in reactions of MoAbs *amv* 5 and *amv* 10, but here mutual influence of conformationally distinct epitopes (neo- and metatope) was involved. In the other MoAbs we observed either no such mutual influence or it was insignificant (20-0 %).

The present results and those reported by Halk *et al.* (1984), Halk (1986) and Hajimorad *et al.* (1990) offer evidence that AMV coat protein contains several immunogenic regions arranged in different conformational structures. We failed to detect epitopes specific for AMV pathotypes, though such evidently do exist (van Vloten-Doting *et al.*, 1968; Hajimorad *et al.*, 1990). AMV is cosmopolitan RNA virus with a presumably high mutation frequency. Our results might suggest that the latter does not affect the open reading frame for coat protein very much.

Acknowledgements. We thank Dr. P. Kontsek, Institute of Virology, Bratislava, for MoAb isotyping, and Mrs. A. Vitazková and Mrs. M. Takáčová for technical assistance.

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