

EFFECT OF *CUCUMBER MOSAIC VIRUS* MONOCLONAL ANTIBODIES ON VIRUS INFECTIVITY AND TRANSMISSION BY *MYZUS PERSICAE*

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SUMMARY

Seven affinity-purified monoclonal antibodies (mAbs) produced against *Cucumber mosaic virus* (CMV) subgroup I and II, were screened for their ability to neutralize virus infectivity *in vitro*. Infectivity was inhibited almost completely when virus preparations were mixed *in vitro* with the homologous mAb prior to inoculation. The most efficient mAbs were 4H10B12 and 6D11D12 for subgroups I and II, respectively. Aggregation of CMV virions following incubation with mAbs was demonstrated using electron microscopy. The ability of the mAbs to block virus transmission by *Myzus persicae* was also tested; both, pre- and post-acquisition treatments of aphids with mAbs significantly decreased virus transmission. Treatment with mAb 6D11D12 before or after feeding on purified virus preparations prevented CMV transmission almost completely.

Key words: *Cucumber mosaic virus*, monoclonal antibodies, transmission by aphids, infectivity neutralization.

INTRODUCTION

Cucumber mosaic virus (CMV) is non-persistently transmitted by numerous species of aphids (Francki *et al.*, 1979). Pre-acquisition starvation of the vector increases efficiency of transmission (Matthews, 1991). The ability of CMV to be transmitted by a vector is determined to a large degree by the properties of the coat protein (CP) (Gera *et al.*, 1979; Chen and Francki, 1990), and *in vitro* heteroencapsidation experiments have shown that the transmissibility of CMV is conferred by the CP (Gera *et al.*, 1979). These studies were followed up by Perry *et al.* (1994), who found that three amino acid mutations in the CP affected transmission

of CMV by *Aphis gossypii*. More recently, Perry *et al.* (1998) found that transmissibility of CMV by *Myzus persicae* requires two mutations in the CP (in positions 25 and 214) in addition to the mutations in positions 129, 162 and 168 reported earlier. *A. gossypii* treated with CMV-specific γ -globulin after feeding on CMV-infected plants, failed to transmit the virus (Fujisawa, 1985).

In Israel, CMV is the most serious virus disease in ornamental and vegetable crops (Gera and Cohen, 1994). The present research was initiated to obtain further information on aphid transmission of the virus. Here we report on the *in vitro* ability of monoclonal antibodies (mAbs) against CMV to neutralize virus infectivity and block virus transmission by *M. persicae*.

MATERIALS AND METHODS

Plant material and virus culture. CMV-Fny (subgroup I) provided by P. Palukaitis from the Cornell plant virus collection, and the local strain CMV-T (subgroup II) (Gera *et al.*, 1978), were used. The viruses were propagated through single-lesions obtained from mechanically inoculated leaves of *Vigna sinensis* prior to use and subsequently maintained in *Cucumis sativus* or *Nicotiana tabacum* L. Xanthi-nc. CMV-T and Fny were purified from *N. tabacum* L. Xanthi-nc following the method of Scott (1963), modified by Gera *et al.* (1979). Purified virus was used fresh or stored at -80°C.

Antibodies. The production and characterization of CMV mAbs in mice was described recently (Hsu *et al.*, 2000). The mAbs used were 4H10B6, 4H10B12 and 6D11B9 which identified all members of CMV in subgroup I but did not react with CMV strains in subgroup II, and 7B3D3, 7B3D9, 6D11D12 and 60B3E7 which reacted with CMV strains in subgroup II, but did not react with CMV strains in subgroup I (Hsu *et al.*, 2000). Antibodies were screened in order to identify those with high-affinity that neutralized CMV infectivity and blocked transmission by aphids. The mAbs were purified from ascitic fluid by affinity chromatography

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on Protein A-Sepharose CL-4B (Sigma P 3391) and dialysed against 0.1% (w/v) NaN_3 in PBS (Saunal *et al.*, 1993). We used CMV polyclonal antibodies produced in our laboratory (Gera *et al.*, 1978) and TMV mAb29, kindly provided by R. Fischer, Institut für Biologie, Aachen, Germany.

Virus neutralization tests. Antibodies against CMV (final concentration $10 \mu\text{g ml}^{-1}$) were mixed with freshly purified CMV (final concentration $1 \mu\text{g ml}^{-1}$) and incubated for one hour at 4°C . *Chenopodium amaranticolor* plants predested with carborondum, were used for the determination of local lesion numbers in a half-leaf assay. Preparations of γ -globulin against CMV and purified mAbs against TMV, with similar concentration were used as positive and negative controls, respectively.

Agar gel double diffusion. Agar gel diffusion tests were done in 55 mm Petri dishes containing a 4 mm layer of 0.75% Bactro agar in 0.85% NaCl and 0.02% sodium azide. A virus concentration of $50 \mu\text{g ml}^{-1}$ and mAbs diluted 1:10 and 1:50 were used. Polyclonal antibodies against CMV diluted 1:50 were used as a positive control.

Electron microscopy. Purified CMV-T ($1 \mu\text{g ml}^{-1}$) was mixed with subgroup II-specific mAbs ($10 \mu\text{g ml}^{-1}$) and incubated at 4°C for one hour. The antigen-antibody mixture was then centrifuged for 10 min at 15,000 rpm. Samples from the supernatant were applied to carbon-coated grids negatively stained with 20 g l^{-1} uranyl acetate and examined in a JEOL 100CX II electron microscope. Particle counts were made at an instrument magnification of $\times 40,000$ using the binoculars ($\times 8$ magnification), on ten fields on each of seven squares on each of two duplicate grids.

Aphid transmission. *M. persicae* (Sulz.) was used for transmission tests. A clone originating from a single female was reared on healthy plants of Japanese radish (*Raphanus sativus*). Nonviruliferous aphids, starved for 2-3 h, were allowed to probe from a purified preparation of CMV-T ($1 \mu\text{g ml}^{-1}$ in 0.05 M PBS containing 10% sucrose) through a parafilm membrane for 60 s (Gera *et al.*, 1978). After probing, two aphids were placed on each test plant (*C. sativus* Bet alpha or *N. tabacum* L. Xanthi-nc). To determine whether mAbs directed against CMV block CMV transmission by aphids, pre- and post-acquisition treatments with antibodies were performed. In the pre-acquisition tests, aphids were allowed to probe first on solutions of mAbs ($100 \mu\text{g ml}^{-1}$ in PBS containing 10% sucrose) and

then on a purified virus preparation ($1 \mu\text{g ml}^{-1}$). For the post-acquisition tests, aphids were allowed to probe first on a purified virus preparation and then on solutions of mAbs, before being placed on test plants. The aphids were allowed to remain on the test plants for 2 h (inoculation access feeding), after which plants were sprayed with nicotine and maintained in the greenhouse for symptom development. A preparation of rabbit γ -globulins against CMV and purified mAbs against TMV in PBS containing 10% sucrose, with similar concentrations were used as a control for pre- and post-acquisition treatments.

RESULTS

Virus neutralization tests. As expected, the subgroup-specific antibodies all neutralized infectivity in homologous tests of mechanical and insect transmission. Subgroup I-specific mAbs neutralized virus infectivity (81 to 86%) as did purified rabbit γ -globulins against CMV (82%) when tested with isolate Fny (Table 1). Subgroup II-specific mAbs inhibited CMV-T to a level of 66 to 87%. The inhibition by mAbs 7B3D9, 6D11D12 and 60B3E7 was significantly higher than with purified rabbit γ -globulins (63%) (Table 1). The mAbs against TMV gave only slight inhibition of infectivity (6-9%). The most efficient mAbs were 4H10B12 for subgroup I, and 6D11D12 for subgroup II.

Analysis of mAb binding by electron microscopy and gel double diffusion. Following incubation of CMV-T virions with subgroup II-specific mAbs, we observed clumping or aggregation of particles in the electron microscope (not shown). After low-speed centrifugation, the number of CMV-T particles observed in the supernatant was significantly decreased following one hour incubation with subgroup II-specific mAb or purified rabbit γ -globulins against CMV. A very low non-specific aggregation was observed following incubation with purified rabbit γ -globulins against TMV (Table 2). Partial decoration of particles was observed when a concentration of 100 ng ml^{-1} of mAbs or γ -globulins against CMV was used (not shown). No aggregation or decoration was observed following incubation with 10 ng ml^{-1} .

In agar-gel-diffusion tests, clear precipitation lines were obtained only between CMV-T and CMV-Fny ($50 \mu\text{g ml}^{-1}$) with polyclonal antibodies diluted 1:50. No reactions were obtained between CMV-T and CMV-Fny with the homologous mAbs diluted 1:10 and 1:50 (not shown).

Table 1. Effect of monoclonal antibodies^a against *Cucumber mosaic virus* (CMV) on the infectivity of purified virus.

Treatment with	CMV-Fny		CMV-T	
	Infectivity ^b	% inhibition	Infectivity	% inhibition
Untreated	32.0 (± 5.5) ^a ^c	–	32.0 (± 3.5)a	–
γ-globulin against CMV	7.0 (± 1.5)b	82	12.0 (± 2.5)b	63
mAb against TMV	30.0 (± 7.0)a	6	29.0 (± 6.0)a	9
Subgroup I specific mAbs				
4H10B6	6.0 (± 2.5)b	81	32.0 (± 4.5)a	0
6D11B9	6.5 (± 1.5)b	80	30.0 (± 1.5)a	6
4H10B12	4.5 (± 2.0)b	86	31.0 (± 3.5)a	2
Subgroup II specific mAbs				
7B3D3	29.0 (± 3.6)a	9	11.0 (± 3.6)b	66
7B3D9	28.0 (± 2.0)a	13	7.5 (± 2.0)bc	77
6D11D12	30.0 (± 1.5)a	6	4.0 (± 1.5)c	87
60B3E7	28.0 (± 3.5)a	13	6.0 (± 2.5)c	81

^a Monoclonal antibodies and purified CMV were used at a concentration of 10 µg ml⁻¹ and 1 µg ml⁻¹, respectively.

^b Number of local lesions per *C. amaranticolor* half-leaf, average from 8-10 leaves, and standard deviation.

^c Values in the same column followed by the same letter are not significantly different. (Tukey's honest significant difference test, $P = 0.05$).

Aphid transmission. The effect of antibody treatment on the transmission of CMV by aphids is summarized in Table 2. Both pre- and post-acquisition treatments with mAbs or purified rabbit γ-globulins against CMV, significantly decreased virus transmission by *M. persicae*. However, post-acquisition treatment with antibodies resulted in significantly higher inhibition of virus transmission than the reverse. MAbs 6D11D12 and 4H10B6 were the most efficient. Purified rabbit γ-globulins against TMV showed only a very low nonspecific inhibition of virus transmission (Table 3).

DISCUSSION

Neutralization of infectivity by immune serum is a test that allows differentiation of viruses or virus strains. The technique has been widely used with animal viruses

(Morgan, 1945). Although not extensively utilized in studies of plant viruses, neutralization has become one of the most important applications in medical virology (Meyer *et al.*, 1994). It provides a means not only for identification of a virus but also protective immunity in vaccination. Only a limited number of reports regarding plant virus neutralization are described in the literature (Gold and Duffus, 1967; Rochow and Ball, 1967; Duffus and Gold, 1969; Liu and Black, 1978; Hsu *et al.*, 1984; Aebig *et al.*, 1987). This is mainly due to the lack of sensitive and precise assays for measurement of virus infectivity and nonspecific interference with infectivity by proteins present in sera (Rappaport and Siegel, 1955; Rappaport *et al.*, 1957).

MAbs prepared against TMV were found to reduce TMV infectivity strongly when they were incubated with the virus prior to inoculation (Dietzgen, 1986; Fischer, 1990). Similar results were obtained with mAbs prepared against CMV (Gu *et al.*, 1987).

Table 2. Number of *Cucumber mosaic virus* (CMV) particles observed by electron microscopy in the supernatant after one hour incubation with poly- and mono-clonal antibodies, followed by low speed centrifugation.

Treatment with	Number of single CMV-T particles ^a at antibody concentration ($\mu\text{g ml}^{-1}$)	
	0.2	1
Untreated	84.2 (15.6) ^b	
γ -globulin against TMV	74.4 (\pm 10.5)a	70.8 (\pm 8.8)a
mAb 7B3D3	11.2 (\pm 2.4)b	5.6 (\pm 1.2)b
mAb 7B3D9	14.6 (\pm 2.8)b	9.6 (\pm 1.8)b
mAb 6D11D12	11.5 (\pm 1.8)b	5.3 (\pm 1.2)b
γ -globulin against CMV	10.3 (\pm 1.6)b	6.1 (\pm 1.4)b

^a Particle counts were made at an instrument magnification of \times 40,000 in viewing fields of 177 mm². Data are average from two experiments.

^b Values in the same column followed by the same letter are not significantly different. (Tukey's honest significant difference test, $P = 0.05$).

The mechanism of neutralization has not been fully explored. Binding of antibodies might alter the surface configuration of virions leading to interference with virus uncoating and release of nucleic acids from protein coats within plant cells (Saunal *et al.*, 1993). Loss of infectivity measured by local lesion assay may also be due to aggregation of virions by antibodies, giving decreased numbers of infectious units. To test these hypotheses, the effect of antibody treatment on virion configuration and aggregation was analyzed. The number of virus particles observed in the EM, following antibody treatment significantly decreased with increase of mAb concentration. Partial decoration of particles was also observed. It is not clear however, why the mAbs showed no reactivity by Ouchterlony immunodiffusion experiments. These results indicate that besides virions aggregation, virus inactivation could also be attributed to covering surface epitopes essential for the host pathogen interaction. The binding of mAbs might inhibit the virus entering the plant cell or interfere with virus disassembly (Saunal *et al.*, 1993).

The question of how nonpersistently transmitted virus, once acquired by aphids, is inoculated has been a subject of considerable speculation. Transmission characteristics first suggested that virus particles are carried as stylet contaminants and inoculated when scoured off

Table 3. Effect of monoclonal antibodies against *Cucumber mosaic virus* (CMV) on the transmission of the virus by *M. persicae*.

Probing sequence	Transmission ^a	
	(no./total)	(%)
Purified CMV-T only	42/50 ^b	84
Purified CMV-T, then sucrose	17/20a	85
Purified CMV-T, then γ -globulin against TMV	16/20a	80
Purified CMV-T, then γ -globulin against CMV	4/40b	10
Purified CMV-T, then mAb 7B3D3	8/40b	20
Purified CMV-T, then mAb 7B3D9	7/40b	17
Purified CMV-T, then mAb 6D11D12	4/40b	10
γ -globulin against TMV, then purified CMV-T	15/20a	75
γ -globulin against CMV, then purified CMV-T	8/40b	20
mAb 7B3D3, then purified CMV-T	12/40b	30
mAb 7B3D9, then purified CMV-T	10/40b	25
mAb 6D11D12, then purified CMV-T	8/40b	20

^a Denominator, number of plants used; numerator, number of plants infected, with two aphids per test plant.

^b Values followed by the same letter are not significantly different (Student- Newman- Keuls', $P = 0.05$).

in the process of probing (Hoggan, 1933). The ingestion-egestion hypothesis (Harris, 1977) postulated that virions in the cibarium and food canal were inoculated when aphids reverse the flow of the cibarial pump, which primarily functions in ingestion. Insects given virus acquisition followed by antibody feeding transmitted less than those fed in a reverse order (Table 3). This higher loss of infectivity, could be due to freshly ingested antibody molecules that are more easily available to virions causing the production of virus-antibody complexes which interfere with the binding of virus to receptor sites of aphid mouthparts or virus ingestion. In addition, low transmission could also be attributed to

repeated probing which leads to lower transmissibility, a common feature of stylet-borne aphid-transmitted viruses (Harris, 1977). A short acquisition access feeding followed by immediate inoculation feeding will result in a higher CMV transmission. At the same time, the fate of antibodies in the insect mouthparts is not clearly understood. Antibodies could be degraded by proteolytic enzymes, inactivated by inhibitors or even absorbed into the digestive system, all processes that would make antibody binding less efficient resulting in less inhibition when antibodies are fed first.

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REFERENCES

- Aebig J.A., Jordan R.L., Lawson R.H., Hsu H.T., 1987. Immunochemical and biological properties of a mouse monoclonal antibody reactive to *Prunus* necrotic ringspot *Illarivirus*. *Intervirology* **28**: 57-64.
- Chen B., Francki R.I.B., 1990. Cucumovirus transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. *Journal of General Virology* **71**: 939-944.
- Dengleng G., Wenbin C., Laiti Y., Chengrun G., Liangyi K., Bingshen Q., Xiuhua Z., Bo T., Jing C., Chengliang Z., 1987. Production and characterization of monoclonal antibodies to tobacco mosaic virus. *Acta Microbiologica Sinica* **27**: 128-133.
- Dietzgen R.G., 1986. Immunological properties and biological function of monoclonal antibodies to tobacco mosaic virus. *Archives of Virology* **87**: 73-86.
- Duffus J.E., Gold A.H., 1969. Membrane feeding and infectivity neutralization used in serological comparison of potato leaf roll and beet western yellows viruses. *Virology* **37**: 150-153.
- Fischer R., 1990. Herstellung, Charakterisierung und Klonierung der Immunglobulingene von TMV neutralisierenden Antikörpern. Ph.D-thesis. University of Tübingen.
- Francki R.I.B., Mossop D.W., Hatta T., 1979. Cucumber mosaic virus. CMI/AAB Description of plant viruses, no. 231.
- Fujisawa I., 1985. Aphid transmission of turnip mosaic virus and Cucumber mosaic virus 2. Transmission from virus mixture. *Annals of the Phytopathology Society of Japan* **51**: 562-568.
- Gera A., Cohen J., 1994. Virus diseases of new ornamental crops. *Israel Agresearch* **7**: 127-137.
- Gera A., Loebenstein G., Raccach B., 1978. Detection of Cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay. *Virology* **86**: 542-545.
- Gera A., Loebenstein G., Raccach B., 1979. Protein coats of two strains of Cucumber mosaic virus affect transmission by *Aphis gossypii*. *Phytopathology* **69**: 396-399.
- Gold A.H., Duffus J.E., 1967. Infectivity neutralization - a serological method as applied to persistent viruses of beets. *Virology* **31**: 308-313.
- Gu D.F., Chen W.B., Yi L.T., Gong C.R., Cang L.Y., Qiu B.S., Zang X.H., Tian B., Chen J., Zang C.L., 1987. Production and characterization of monoclonal antibodies to cucumber mosaic virus. *Acta Microbiologica Sinica* **27**: 128-133.
- Harris K.F., 1977. An ingestion-egestion hypothesis of noncirculative virus transmission by aphids. In: Harris K.F., Maramorosch K. (eds.). *Aphids as virus vectors*, pp. 165-220. Academic Press, New York.
- Hoggan I.A., 1933. Some factors involved in aphid transmission of the Cucumber mosaic virus to tobacco. *Journal of Agriculture Research* **47**: 689-704.
- Hsu H.T., Aebig J., Rochow W.F., 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. *Phytopathology* **74**: 600-605.
- Hsu H.T., Barzuna L., Hsu Y.H., Bliss W., Perry K.L., 2000. Identification and subgrouping of *Cucumber mosaic virus* with mouse monoclonal antibodies. *Phytopathology* **90**: 615-620.
- Liu H.Y., Black L.M., 1978. Neutralization of infectivity of potato yellow dwarf virus and wound tumor virus assayed on vector-cell monolayers. *Phytopathology* **68**: 1243-1248.
- Matthews R.E.F., 1991. *Plant virology*. Academic Press. San Diego, CA.
- Meyer H., Osterrieder N., Czerny C.A., 1994. Identification of binding sites of neutralizing monoclonal antibodies on the 14-kDa fusion protein of orthopox viruses. *Virology* **200**: 778-783.
- Morgan I.M., 1945. Quantitative study of the neutralization of western equine encephalomyelitis virus by its antiserum and the effect of the complement. *Journal of Immunology* **50**: 359.
- Perry K.L., Zhang L., Palukaitis P., 1998. Amino acid changes in the coat protein of Cucumber mosaic virus differentially affect transmission by aphids *Myzus persicae* and *Aphis gossypii*. *Virology* **242**: 204-210.
- Perry K.L., Zhang L., Shintaku M.H., Palukaitis P., 1994. Mapping determinants in Cucumber mosaic virus for transmission by *Aphis gossypii*. *Virology* **205**: 591-595.

Rappaport I., Siegel A., 1955. Inactivation of tobacco mosaic virus by rabbit antiserum. *Journal of Immunology* **74**: 106-116.

Rappaport I., Siegel A., Owen R.D., Wildman S.G., 1957. A comparative study of antibody inactivation of tobacco mosaic virus. *Journal of Immunology* **78**: 259-261.

Rochow W.F., Ball E.M., 1967. Serological blocking of aphid transmission of barley yellow dwarf virus. *Virology* **33**: 359-362

Saunal H., Witz J., Van Regenmortel M.H., 1993. Inhibition of in vitro cotranslational disassembly of tobacco mosaic virus by monoclonal antibodies to the viral coat protein. *Journal of General Virology* **74**: 897-900.

Scott H., 1963. Purification of Cucumber mosaic virus. *Virology* **20**: 103-106.

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