J. Fac. Agr. Hokkaido Univ., Vol. 64, Pt. 2 : 107~115 (1990)

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RICE DWARF VIRUS

Jumanto HARJOSUDARMO, Kazusato OHSHIMA
Ichiro UYEDA and Eishiro SHIKATA

Department of Botany, Faculty of Agriculture
Hokkaido University, Kita-ku, Kita-9, Nishi-9
Sapporo 060, Japan

Received September 7, 1989

Introduction

Serological techniques had been promoted for rapid and sensitive detection of plant viruses using microprecipitine, latex flocculation, passive haemaglutination or enzyme linked immunosorbent assays (ELISA) to manage plant virus diseases[10] in relation to polyclonal and monoclonal antibodies. Rice dwarf virus (RDV) is one of the major rice diseases in Japan, and persistently transmitted by *Nephotettix cinticeps* Uhler.[5]. Although RDV have been extensively studied, such as morphology[12], biochemical properties[13], and nucleic acid sequences of its genome[14], there is less information on serological aspect especially on monoclonal antibodies (MoAbs).

In this paper, we reported the production, characteristics of MoAbs to RDV and their use for detecting the virus in crude sap or individual insect by using direct double antibody sandwich (DAS) ELISA.

Materials and Methods

Virus purification: RDV infected plants were prepared by inoculating healthy rice plants (cv. Norin 8) using viruliferous *N. cinticeps* that had been fed on the diseased plants for a week, then kept it on healthy plants for a week before inoculation. Virus purification were done according to the method described by Uyeda and Shikata[12], except for Driselase treatment.

Polyclonal antibody: Rabbit anti-RDV polyclonal antibody (PoAb) had a titer of 5^5 determined by microprecipitine test, were used in this experiment.

Virus antigen in crude sap: One gram of virus infected rice leaves were homogenized with 9 ml of 0.02 M phosphate buffer saline, pH 7.4 containing 0.05% Tween-20 (PBS-T). Crude sap from healthy leaves were made by same procedure, as a control.

Immunization and hybridization: The spleens of 4 weeks BALB/c mice were immunized by a single injection with 30 μg of purified RDV in 100 μl of 0.01 M

phosphate buffer, pH 6.0, four days before hybridization. The hybridization was conducted according to Ohshima *et al.*[8], except the spleen and myeloma cell ratio was used 13 : 1, respectively.

Screening : Two weeks after fusion, hybridomas were screened for the present of antibodies by four different ELISA procedures (see ELISA procedures). Screening was done twice. Anti-mouse peroxidase conjugate in 3000 times dilutions (Cappel Inc.) was used as a probe in the first screening. Hybridomas which gave positive response were multiplied in petridishes of 6 cm diameter, and the culture fluids were screened again (second screening) using same methods, except the probe was anti-mouse alkaline phosphatase conjugate in 1000 times dilutions (Zymed Lab.). Some of the hybridomas showing positive response in second screening were cloned twice by limiting dilution method. The others were freezed and stored in liquid nitrogen. As first and second screening data showed the similar response pattern, only second secreening data were presented in this paper.

Production of ascitic fluids : An amount of 0.5-1.0 x 10^6 cells of each MoAb -secreting hybridomas were injected into peritoneal cavity of pristane-primed BALB/c mice. About two weeks after injection, the ascitic fluids were collected for immunoglobulin preparation.

Preparation of immunoglobulin (Ig) : The Ig from both PoAb and MoAb were purified by ammonium sulphate precipitation followed by Wathman DE-32 column chromatography. Ig types of MoAbs were determined by AAI-ELISA (procedure 2) using goat anti-mouse Ig, which is specific to IgG (gamma chain), IgM (mu chain) and IgA (alpha chain) were purchased from Cappel Inc. Titers were also determined by the same procedure. Alkaline phosphatase (Boehringer Mannheim, Grade 1) was used for conjugation with Ig using method described by Clark and Adams[2].

ELISA procedures : The procedures of ELISA used in this experiments were shown in Table 1. Procedure 1 and 2 (AAI-ELISA, Antigen Adsorption Indirect -ELISA) were followed by the method described by Crook and Payne[1]. The wells of polystyrene plates (Nunc, Denmark) were coated with 1 μg/ml of purified virus in 0.02 M phosphate buffer saline at pH 7.4 (PBS) for procedure 1, instead in 0.05 M sodium carbonate-bicarbonate buffer at pH 9.6 (SCB) for procedure 2, and incubated at 4°C overnight. Plates were washed three times with PBS-T and antibodies from the hybridomas were added for 3 hr at 37°C. After washing, the alkaline phosphatase labeled anti-mouse Ig conjugate (Zymed Lab.) was used as a probe at 1000 times dilutions in PBS-T and incubated for 3 hr at 37°C. The substrate (p-nitrophenyl phopsphate) was then added after washing, and absorbance value was readed at A_{415} with a Corona MTP-100 spectrophotometer after 15 to 60 minutes incubation. Procedure 3 and 4 (IDAS-ELISA, Indirect Double Antibody Sandwich-ELISA) were followed by the procedure described by Massal-

TABLE 1. Different ELISA procedures used in this experiment

Procedures	ELISA[b)]	1st step (trapping)	2nd step	3rd step	4th step
1.	AAI	antigen (in PBS)	culture sap (MoAb)	mouse Ig-EC[a)]	
2.	AAI	antigen (in SCB)	culture sap (MoAb)	mouse Ig-EC	
3.	IDAS	PoAb	antigen (purified)	culture sap (MoAb)	mouse Ig-EC
4.	IDAS	PoAb	antigen (crude sap)	culture sap (MoAb)	mouse Ig-EC
5.	DAS	MoAb	antigen	MoAb Ig-EC	

a) EC =enzyme conjugate
b) AAI =Antigen Adsorption Indirect
IDAS =Indirect Double Antibody Sandwich
DAS =Double Antibody Sandwich
Procedures 1, 2, 3 and 4 were used for screening

ski and Harrison[7]. In this procedures, plates were first coated with 2 μg/ml of PoAb in SCB for 3 hr at 37°C. Afrer washing with PBS-T ? the antigens, (the purified virus for procedure 3, and the crude sap from infected leaves for procedure 4) were added. The subsequent steps were the same as procedures 1 and 2. Procedure 5 (DAS-ELISA, Double Antibody Sandwich-ELISA) were followed by the method described by Clark and Adams[2].

Detection of RDV antigen in individual insect: The insect had been fed on RDV infected plants for a week and confined on the healthy rice plants for three weeks. They were transfered into eppendorf tube individually, and were crushed by glass rods in 500 μl of PBS-T. The extracts were used for DAS-ELISA.

Results

Production and screening of antibody-secreting hybridomas.

After cell fusions eight times, 23 out of 352 wells grew hybridomas (6.5%). Based on the response of hybridomas in four ELISA screening procedures tested, they divided into 6 groups as shown in Table 2. Group 1 includes 12 out of 23 hybridomas (52.2%) that reacted in all four ELISA procedures. Four hybridomas (17.4%, group 5) reacted in IDAS-ELISA (procedure 3 and 4) only, 3 (13%, group 3) in AAI and IDAS-ELISA (procedure 2, 3, 4), 2 (8.7%, group 6) in IDAS-ELISA (procedure 3) only. Each one hybridoma was screened in group 2 and 4. Eight hybridomas selected from group 1 and 5 were cloned and ascitic fluids were produced. The absorbance values of the purified MoAbs in four screening procedures were shown in Table 3. The reactions correspond to the result

TABLE 2. Grouping of hybridomas secreting RDV-antibody

Group	No. of hybridomas	ELISA procedures[a] 1	2	3	4	%[b]
1.	RD (1)-1 C10	0.49(+)	0.74(+)	1.48(+)	1.45(+)	52.2
	F03	0.47(+)	0.66(+)	1.17(+)	1.02(+)	
	F08	0.41(+)	0.59(+)	0.84(+)	0.96(+)	
	(6)-3 C12	0.61(+)	0.91(+)	1.58(+)	1.05(+)	
	(7)-1 B05	0.62(+)	0.89(+)	1.26(+)	0.94(+)	
	E05	0.59(+)	0.87(+)	1.26(+)	0.86(+)	
	G04	0.28(+)	0.47(+)	0.86(+)	0.56(+)	
	(7)-2 G04	0.41(+)	0.69(+)	1.03(+)	0.76(+)	
	(7)-3 A09	0.22(+)	0.33(+)	0.64(+)	0.41(+)	
	(8)-1 E031	0.35(+)	0.73(+)	1.17(+)	0.87(+)	
	(8)-2 F09	0.27(+)	0.45(+)	0.84(+)	0.56(+)	
	(9)-2 C09	0.47(+)	0.64(+)	1.46(+)	1.00(+)	
2.	(6)-2 H10	0.25(+)	0.34(+)	0.75(+)	0.17(−)	4.3
3.	(6)-1 B03	0.14(−)	0.25(+)	0.64(+)	0.30(+)	13.0
	(7)-2 G10	0.13(−)	0.32(+)	0.67(+)	0.42(+)	
	(8)-2 A05	0.15(−)	0.32(+)	0.69(+)	0.46(+)	
4.	(7)-1 F07	0.15(−)	0.32(+)	0.50(+)	0.05(−)	4.3
5.	(1)-1 B10	0.05(−)	0.11(−)	0.21(+)	0.26(+)	17.4
	(7)-1 C10	0.08(−)	0.11(−)	0.75(+)	0.40(+)	
	(8)-1 D03	0.06(−)	0.14(−)	0.50(+)	0.40(+)	
	(8)-2 G111	0.05(−)	0.12(−)	0.50(+)	0.33(+)	
6.	(7)-1 H05	0.06(−)	0.07(−)	0.55(+)	0.04(−)	8.7
	(8)-1 E08	0.11(−)	0.02(−)	0.56(+)	0.04(−)	

a) ELISA procedures in detail were shown in TABLE 1.

b) Numbers of antibody secreting hybridomas belong to the group/number of antibody secreting hybridomas

obtained from the second screening (Table 2), except for the MoAb RD (1)-1 B10 which belong to group 5, it was reacted in all four ELISA procedures after purification from ascitic fluids.

Characteristics of eight purified MoAbs.

As shown in Table 4, seven hybridomas secreted IgG, and only RD (9)-2 C09 characterized as IgM. Two hybridomas, suchs as RD (1)-1 C10 and RD (1)-1F08, gave high titers, at 5^6 against RDV, while RD (8)-2 F09 showed very low titers.

Reaction of RDV-MoAbs in DAS-ELISA.

For detecting the virus antigen in crude sap, attempt to use MoAbs for both

TABLE 3. Reactions of MoAbs purified from ascitic fluids in four ELISA screening procedures

Hybridomas (MoAbs)	ELISA procedures			
	1	2	3	4
RD (1)-1 B10	0.26	0.89	0.34	0.38
(1)-1 C10	2 <	2 <	2 <	2 <
(1)-1 F03	2 <	2 <	1.43	1.45
(1)-1 F08	2 <	2 <	2 <	1.06
(6)-3 C12	2 <	2 <	2 <	1.89
(7)-1 G04	0.35	0.41	0.83	0.76
(8)-2 F09	0.36	0.48	1.26	1.23
(9)-2 C09	0.83	1.09	1.83	1.41

Hydrolysis time was 60 min.

TABLE 4. Titers of RDV-MoAbs against purified RDV preparations

MoAbs	Titer (reciprocal)	Ig types[a)]
RD (1)-1 B10	5^2	IgG
(1)-1 C10	5^6	IgG
(1)-1 F03	5^5	IgG
(1)-1 F08	5^6	IgG
(6)-3 C12	5^4	IgG
(7)-1 G04	5^4	IgG
(8)-2 F09	5^1	IgG
(9)-2 C09	5^4	IgM

a) 5°, indicated the MoAbs were used at the concentration of 1 μg/ml

trapping and second antidody in DAS-ELISA (procedure 5) was made. Positive response, as shown in Table 5, was obtained in MoAbs from RD (1)-1 C10, RD (9)-2 C09 and RD (8)-2 F09 hybridomas, in which RD (1)-1 C10 reacted in the highest response, with low background.

Comparison of serological response by RD (1)-1 C10 MoAb and PoAb in DAS-ELISA.

As shown in Fig. 1, RD (1)-1 C10 could detect RDV up to 16 ng purified virus and up to 10^3 dilution of crude sap, but PoAb gave a lower absorbance value at the same conditions.

TABLE 5. Reactions of RDV-MoAbs in DAS-ELISA

MoAbs[a]	MoAb-EC X200 Disease	X200 Healthy	X200 PBS	X400 Disease	X400 Healthy	X400 PBS[b]
RD (1)-1 B10	0.17	0.05	0.00	0.14	0.05	0.00
(1)-1 C10	2 <	0.03	0.00	2 <	0.02	0.00
(1)-1 F03	0.16	0.05	0.00	0.08	0.06	0.00
(1)-1 F08	0.21	0.06	0.00	0.11	0.04	0.00
(6)-3 C12	0.12	0.07	0.00	0.05	0.00	0.00
(7)-1 G04	0.08	0.01	0.00	0.06	0.00	0.00
(8)-2 F09	0.44	0.11	0.00	0.37	0.10	0.00
(9)-2 C09	0.42	0.00	0.00	0.29	0.05	0.00

a) 5 μg/ml MoAbs were used for coating

b) Disease.; crude sap from the diseased leaves, H.; healthy

MoAb-EC; monoclonal antibodies enzyme conjugate

Hydrolysis times was 60 min.

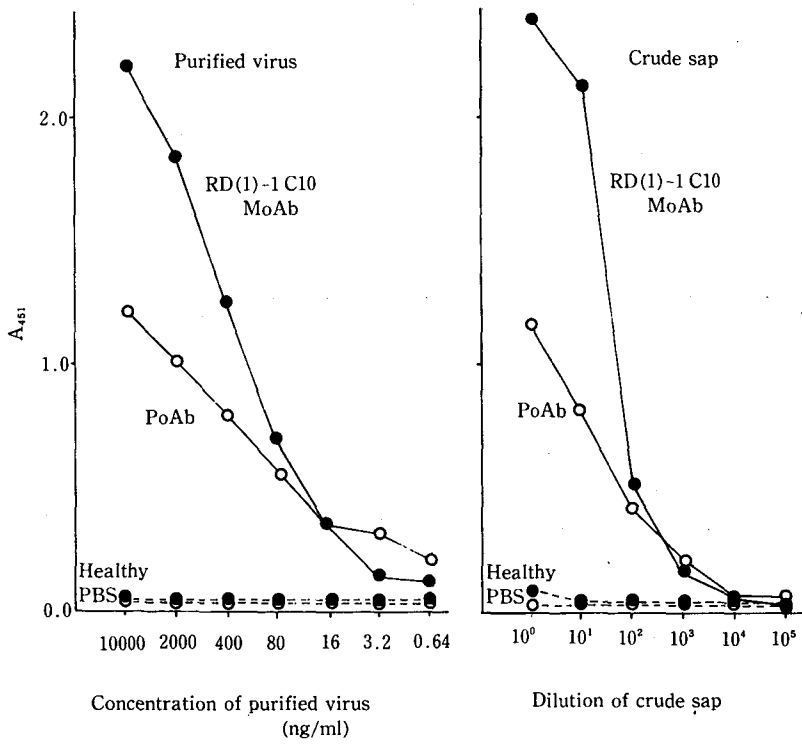

Fig. 1. Response of RD(1)-1 C10 MoAb and PoAb in DAS-ELISA

Detection of RDV in individual insect by DAS-ELISA.

Five from 20 wells gave positive responses for the viral antigen in individual insects where absorbance values were ranging from 1.25 to 2.09, with little background when RD (1)-1 C10 was employed. However, lower absorbance values were obtained when PoAb was employed in the same samples. Total 25% of the insects tested were shown to be infected (Table 6). The positive insects detected by MoAb-ELISA were also shown to be positive by PoAb-ELISA.

Discussion

Using PoAb, practical application of ELISA for detecting plant viruses has

TABLE 6. Detection of RDV in individual insect using RD(1)-1 C10 MoAb and PoAb by DAS-ELISA

Insect no.	Absorbance (A_{415}) RD (1)-1 C10 MoAb	PoAb
1.	0.00(−)	0.01(−)
2.	0.00(−)	0.00(−)
3.	2.01(+)	0.97(+)
4.	0.00(−)	0.01(−)
5.	0.00(−)	0.00(−)
6.	0.00(−)	0.00(−)
7.	0.00(−)	0.02(−)
8.	2.09(+)	0.87(+)
9.	0.00(−)	0.00(−)
10.	0.00(−)	0.00(−)
11.	0.00(−)	0.01(−)
12.	0.00(−)	0.01(−)
13.	1.25(+)	0.82(+)
14.	0.00(−)	0.01(−)
15.	0.00(−)	0.00(−)
16.	1.52(+)	1.02(+)
17.	0.00(−)	0.01(−)
18.	1.31(+)	1.02(+)
19.	0.00(−)	0.01(−)
20.	0.00(−)	0.01(−)
21. control	0.00(−)	0.00(−)
PBS	0.00(−)	0.00(−)

a) 1st antibody (trapping), 5 μg/ml
2nd antibody-enzyme conjugate ×400
Hydrolysis time was 20 min

been performed mostly by DAS-ELISA, because it has an advantage in detecting antigens not only in purified preparations but also in crude saps. Therefore, it is desirable to screen MoAbs which can be applicable to DAS-ELISA. Ohshima *et al.* (personal communication) indicated that most of the MoAbs screened for luteoviruses could not be used for DAS-ELISA, but applicable to AAI-ELISA.

In this study, the authors intended to produce MoAbs that can be practically applicable to DAS-ELISA for RDV in crude sap preparations. Within 23 hybridomas screened, eight MoAbs to RDV were finally obtained and purified. Three of them were reacted with the virus antigen in crude sap by DAS-ELISA using MoAbs for trapping and second antibodies (Table 5), but only RD (1)-1 C10 showed high absorbance values with less background. It was also useful for detecting virus antigen in individual insect by DAS-ELISA (Table 6).

There were many reports of DAS-ELISA using MoAbs for detecting filamentous viruses, i. e. rice stripe virus tenuivirus[10], potato virus Y potyvirus[3], tulip breaking potyvirus[4] and citrus tristeza closterovirus[15]. On the other hand, a few reports have been published on spherical viruses, i. e. tobacco necrotic dwarf luteovirus by Ohshima *et al.*[9]

Maeda *et al.*[6] employed PoAb for trapping antigens as first antibody and used MoAb to cucumber mosaic virus as second antibody in DAS-ELISA. In this way, there is little advantage for routine indexing, because one must prepare both PoAbs and MoAbs against individual viruses.

In this experiment, we succeded to produce a MoAb to RDV that was applicable for DAS-ELISA, which responded much higher than PoAb in crude sap and purified preparation (Fig. 1), or in individual insects. As shown in (Table 6), higher absorbance values and less background than PoAb were obtained when RD (1)-1 C10 MoAb was used to detect viral antigen in leafhopper insect vectors. The fact further supports the diagnostic application of DAS-ELISA using RD (1) -1 C10 MoAb. Detection of RDV in insect vectors could be very useful for establishing the control measure of the disease. As a specific MoAb capable for DAS-ELISA is once established, it has an advantage of its continuous supply for routine diagnostic procedures.

Summary

Monoclonal antibodies to rice dwarf virus (RDV) were produced by fusing the BALB/c mice spleen cells with SP 2/0 Ag-14 myeloma cells. The following ELISA procedures were used to screen the antibody-secreting hybridomas; Antigen Adsorption Indirect (AAI) ELISA using phosphate buffer saline at pH 7.4 or sodium carbonate-bicarbonate buffer at pH 9.6; Indirect Double Antibody sandwich (IDAS) ELISA using crude sap or purified virus.

Eight hybridomas secreting monoclonal antibodies (MoAbs) were selected, in which seven were IgG, and another one was IgM. The titers of purified MoAbs

ranged from 5^1 to 5^6.

The RD (1)-C10 MoAb could be used for first trapping antibody and for the second antibodies is DAS-ELISA and was able to detect viral antigens in up to 16 ng purified virus or diluted crude sap at about 10^3. It was also useful for detecting the viral antigen from an individual leafhopper insect vector, using direct double antibody sandwich (DAS) ELISA. Therefore, that MoAb is a good candidate for RDV diagnostic purpose.

Literature cited

1. Crook, N. E., Payne, C. C.: Comparison of three methods of ELISA for baculovirus. J. Gen. Virol., **46**: 29-37,1980
2. Clark, M. F., Adams, A. N.: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol., **34**: 475-483, 1977
3. Gugerli, P., Fries, P.: Characterization of monoclonal antibodies to potato virus Y and their use for virus detection. J. Gen. Virol., **64**: 2471-2477, 1983
4. Hsu, H. T., Fransen, J. M., van der Hulst, C. T. C., Derks, A. F. L. M., Lawson, R. H.: Factors affecting selection of epitope specificity of monoclonal antibodies to tulip breaking potyvirus. Phytophatology, **78**: 1337-1340, 1988
5. Kitagawa, K., Okuhara,E., Shikata, E.: Production of monoclonal antibodies against rice dwarf virus and characterization of antigen protein with affinity media. J. of Virological Methods, **16**: 217-224, 1987
6. Maeda, T., Sako, N., Inouye, N.: Production of monoclonal antibodies to cucumber mosaic virus and their use in enzyme-linked immunosorbent assay. Ann. Phytopath., Soc. Japan, **54**: 600-605, 1988
7. Massalski, P. R., Harrison, B. D.: Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolate differing in aphid transmissibility. J. Gen. Virol, **68**: 1813-1821, 1987
8. Ohshima, K., Uyeda, I., Shikata, E.: Production and characteristic of monoclonal antibodies to potato leafroll virus. J. Fac. Agr. Hokkaido Univ., **63**: (4): 373-388,1988
9. Ohshima, K., Uyeda, I., Shikata, E.: Charactesization of monoclonal antibodies against tobacco necrotic dwarf virus. Ann. Phytopath. Soc. Japan, **55**: 420-426,1989
10. Omura, T., Takahashi, Y., Shohara, K., Minobe, Y., Tsuchizaki, T., Nozu, Y.: Production of monoclonal antibodies against rice stripe virus for the detection of virus antigen in infected plants and viruliferous insects. Ann. Phytopath. Soc. Japan, **52**: 270-277,1986
11. Shohara, K., Takahashi, Y., Ohshima, N., Shimamora, T., Araki, T.: Abstracs papers. 5th international congres of plant pathology. Kyoto, Japan, 1988
12. Uyeda, I., Shikata, E.: Ultrastructure of rice dwarf virus. Ann. Phytopath. Soc. Japan, **48**: 295-300,1982
13. Uyeda, I., Shikata, E.: Characterization of RNAs synthesized by virion associated transcriptase of rice dwarf virus in vitro. Virus research, (1): 527-532, 1984
14. Uyeda, I., Matsumura, T., Sano, T., Ohshima, K., Shikata, E.: Nucleotide sequence of rice dwarf virus genome segment 10. Proc. Japan Acad. Sci., **63**: 227-230, 1987
15. Vela, C., Cambra, M., Cortes, E., Moreno, P., Miguet, J. G., Perez de San Roman, C., Sanz, A.: Production and characterization of monoclonal antibodies specific for citrus tristeza virus and their use for diagnosis. J. Gen. Virol, **67**: 91-96, 1986