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## PRODUCTION AND CHARACTERISTICS OF MONOCLONAL ANTIBODIES TO POTATO LEAFROLL VIRUS

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### Introduction

Using polyclonal antibodies (PoAbs), it was shown that potato leafroll virus (PLRV), a member of luteovirus, was serologically related to beet western yellows virus (BWYV)<sup>9</sup>, barley yellow dwarf virus (BYDV)<sup>10,11</sup>. Recently, MASSALSKI and HARRISON<sup>9</sup> produced monoclonal antibodies (MoAbs) against British isolate of PLRV, but they could not find MoAb which recognized common epitope among PLRV and BYDV.

Since direct method of "Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)" using PoAbs was introduced by CLARK and ADAMS<sup>2</sup>, various methods of indirect-ELISA were described<sup>3,7,8</sup> using PoAbs. Al MOUDALLAL *et al.*<sup>12</sup> discussed on the application of this method on tobacco mosaic virus using MoAb in combination with PoAb and suggested that two indirect-ELISA procedures possessed high sensitivities. But there was no report on the comparison between indirect-ELISA and DAS-ELISA using MoAbs.

We evaluated various types of direct- and indirect-ELISA and found that individual PLRV MoAbs reacted differently to PLRV, BWYV and BYDV depending on the ELISA procedures. In this paper, we describe reaction characteristics of each MoAb in different ELISA among three luteoviruses; PLRV, BWYV and BYDV.

### Materials and Methods

**Virus isolates.** PLRV and BWYV were maintained in *Physalis floridana*, and BYDV (805 isolate<sup>10</sup>) was maintained in barley (*Hordeum vulgare*) in a green house.

**Preparation of Antigens.** The method described by TAKANAMI and KUBO<sup>12)</sup> for PLRV and BWYV, and by MATSUBARA *et al.*<sup>10)</sup> for BYDV, were employed for antigen preparation.

**Production of monoclonal antibodies.** Four weeks-aged mice were hyperimmunized with the virus preparations. Two hundreds  $\mu\text{g}$  of the purified viruses emulsified with Freund's complete adjuvant were injected twice into the intraperitoneal cavity followed by a booster of two hundreds  $\mu\text{g}$  virus in phosphate buffered saline 4 weeks later. The titers of antisera of mice were detected by ELISA 3 to 4 days after the booster.

Spleen cells were fused with non-secretor SP2/O Ag-14 cells. Exponentially growing SP2/O cells in Dulbecco's modified Eagle's medium (DMEM), consisting of 0.548 g L-glutamine, 200 mg Kanamicine, 150 ml new born calf serum (Flow Lab. Inc.), 16 ml 10% sodium bicarbonate and 9.4 g powdered DMEM (Nissui 2, Nissui Pharmaceutical Co., Ltd.) per liter at pH 7.2, and sterilized by filtration or autoclaved.

Hybridization was carried out by mixing  $3.5 \times 10^8$  spleen cells with  $3.5 \times 10^7$  cells of SP2/O and then fused using 50% polyethylen glycol #1,000 (Kanto Chem. Co., Inc.). Cells were cultured under HAT-DME selective medium (0.1 mM hypoxantine, 0.42  $\mu\text{M}$  aminopterin, 64  $\mu\text{M}$  thymidine) in 96-well NUNCS trays and aliquots of cell suspension were grown in a 37°C humidified incubator in a 5% CO<sub>2</sub> and 94% air atmosphere. After 2, 4 and 10 days fusion, the HAT-DMEM was added. Two weeks after cell fusion, the cell-free culture fluids were screened for the presence of antibodies against PLRV using indirect-ELISA in which the antigens were adsorbed at the first step (see below). Specific antibody secreting hybridomas were cloned by limiting dilution method. Cloning was repeated twice or three times and established hybrids were grown in HT medium (HAT-medium without aminopterin).

**Antigen adsorption indirect-ELISA (AAI-ELISA).** AAI-ELISA was used for detecting specific antibodies for PLRV in cultured fluids. Ninety six wells of polystyrene plates (Nunc, #4-42404) were coated with 1  $\mu\text{g}/\text{ml}$  of a purified preparation of PLRV in 100  $\mu\text{l}$  sodium carbonate buffer (0.05 M, pH 9.6), and incubated overnight at 4°C. Plates were washed with PBS (0.15 M NaCl in sodium phosphate, pH 7.4) containing 0.05% Tween 20 (PBS-Tween) and 100  $\mu\text{l}/\text{well}$  of undiluted cell-free culture fluid was pipetted into the wells. Peroxidase conjugated goat anti-mouse immunoglobulin (IgG + IgA + IgM) was purchased from Cappel Lab., Inc..

Reactions were considered positive when the absorbance readings ( $A_{492}$ ) obtained from the cultured fluids containing antibodies were more than three

times higher than those obtained from the cultured fluids of myeloma SP2/O.

**Determination of immunoglobulin type of MoAbs.** Immunoglobulin (Ig) types of MoAbs were determined by AAI-ELISA using peroxidase conjugated goat anti-mouse Ig which was specific for IgG (gamma — chain specific, Cappel Lab., Inc.), IgM (mu-chain specific, Cappel Lab., Inc.), IgA (alpha-chain specific, Cappel Lab., Inc.).

**Production of ascites fluids.** Ascites fluids was produced by intraperitoneal inoculation of  $5 \times 10^5$  to  $1 \times 10^6$  hybrid cells into synergenic BALB/c mice that 0.5 ml pristane (2, 6, 10, 14-tetramethylpentadecane, Kanto Chem. Co., Inc.) had been injected 2 weeks before inoculation.

**Purification of antibody.** MoAbs were purified from ascites fluids using ammonium sulphate precipitation and DEAE column chromatography (DE 32, Whatman Ltd.).

IgG was precipitated by ammonium sulphate at 1/3 saturation and IgM was at 1/2 saturation. NaCl concentration in sodium phosphate buffer used in the DEAE column chromatography were 0.02 M for IgG and 0.2 M for IgM.

**Procedures of ELISA.** Procedures of ELISA used in this paper are shown in Table 1. Double antibody sandwich ELISA (DAS-ELISA) was followed by the method described in CLARK and ADAMS<sup>2)</sup> and KOENIG<sup>6)</sup>. Antigen adsorption direct-ELISA (AAD-ELISA) and AAI-ELISA were followed by the method of CROOK and PAYNE<sup>3)</sup> and VAN REGENMORTEL and BURCKARD<sup>19)</sup>. Concentration of antibodies used in this experiment was

TABLE 1. Procedures of direct- and indirect-ELISA using monoclonal antibodies, and monoclonal antibodies using in combination with polyclonal antibodies

| Steps of ELISA procedures   | Names of ELISA procedures             |
|-----------------------------|---------------------------------------|
| 1. AG, MoAb-E               | antigen adsorption direct (AAD)       |
| 2. MoAb, AG, MoAb-E         | double antibody sandwich direct (DAS) |
| 3. AG, MoAb, anti-M-E       | antigen adsorption indirect (AAI)     |
| 4. PoAb, AG, MoAb-E         | double antibody sandwich direct       |
| 5. MoAb, AG, PoAb-E         | double antibody sandwich direct       |
| 6. PoAb, AG, MoAb, anti-M-E | double antibody sandwich indirect     |
| 7. MoAb, AG, PoAb, anti-M-E | double antibody sandwich indirect     |

AG: antigen, MoAb: monoclonal antibody, PoAb: polyclonal antibody, E: enzyme, anti-M: goat anti-mouse Ig, anti-R: goat anti-rabbit Ig

5  $\mu\text{g}/\text{ml}$  and alkaline phosphatase conjugated virus specific antibodies were made in our laboratory and alkaline phosphatase conjugated mammalian Ig specific antibodies purchased from Zymed Lab., Inc. were used at 400 folds dilution by PBS-Tween. Plates were washed three times by PBS-Tween and all steps of reaction mixture were performed in 200  $\mu\text{l}/\text{well}$ .

## Results

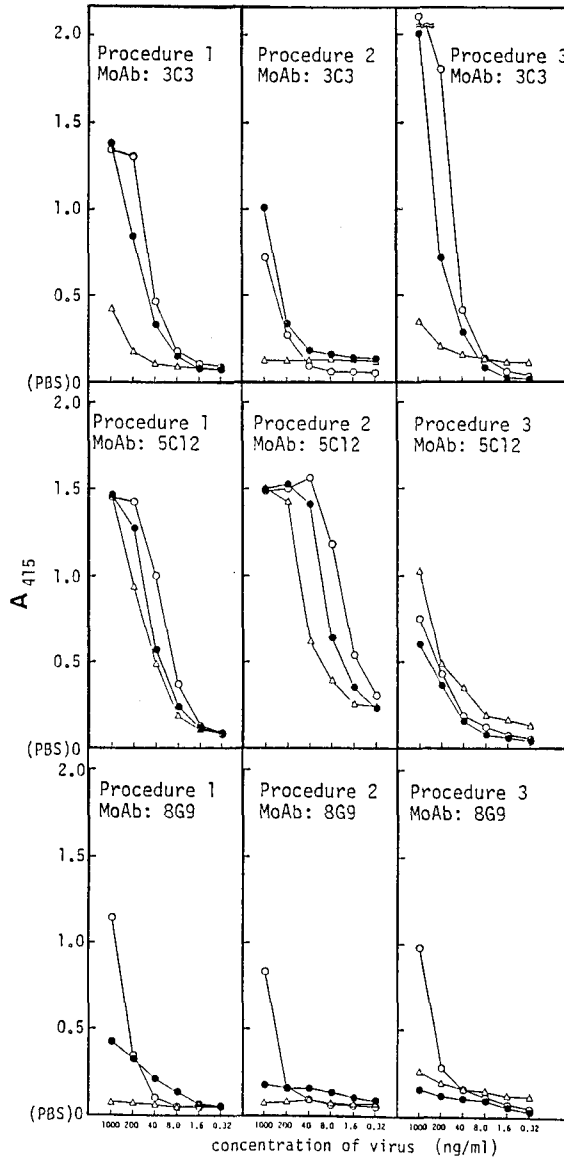
**Production and characterization of monoclonal antibodies.** Fusion experiments were performed to produce MoAbs against PLRV twice. Seventy three out of 720 wells of the first fusion products grew hybrid cells (10.1%), in which cultured fluids of 21 cultured wells (28.8%) reacted positively with PLRV by AAI-ELISA. Growth of hybrid cells in 960 wells of the second fusion products were observed in 527 wells (54.9%), in which cultured fluids of 182 wells (34.5%) reacted positively with PLRV. Nine hybrid cells were cloned by the limiting dilution method and cultured fluids of the two were reacted with healthy *Physalis floridana* extracts.

Characterization of Ig type of seven cell lines secreting anti-PLRV MoAbs were performed by AAI-ELISA (Table 2). It was shown that PLR-3C3, 9D4 were IgG and the remaining five cell lines, PLR-5C12, 6E5, 7F10, 8G9 and 11E8, were IgM.

TABLE 2. Monoclonal antibodies against PLRV and its Ig type

| Monoclonal antibodies | Ig type | Monoclonal antibodies | Ig type |
|-----------------------|---------|-----------------------|---------|
| 3C 3                  | IgG     | 8G 9                  | IgM     |
| 5C12                  | IgM     | 9D 4                  | IgG     |
| 6E 5                  | IgM     | 11E 8                 | IgM     |
| 7F10                  | IgM     |                       |         |

**ELISA using monoclonal antibodies.** Three clones, 3C3, 5C12 and 8G9, were picked up for further studies. Since other four clones reacted similarly to the three clones among PLRV, BWYV and BYDV on AAI-ELISA (screening procedure). Three clones were examined by three different procedures of ELISA such as procedure 1 (AAD-), procedure 2 (DAS-) and procedure 3 (AAI-), as shown in Table 1. The results were shown in Fig. 1. It was shown that remarkably different reactions were detected in the three ELISA procedures for each MoAb. Reaction of 3C3 with the three viruses in AAI-ELISA was relatively high but that in DAS-ELISA was low. 3C3



**Fig. 1.** Detection of the three luteoviruses by direct- and indirect-ELISA using PLRV monoclonal antibodies. Steps of ELISA procedures are shown in Table 1. The first and second antibodies were incubated for 3 hr at 37°C. The antigen of purified virus, PLRV (●---●), BWYV (○---○) and BYDV (△---△) were incubated for overnight at 6°C. Hydrolysis time was 1 hr at 37°C.

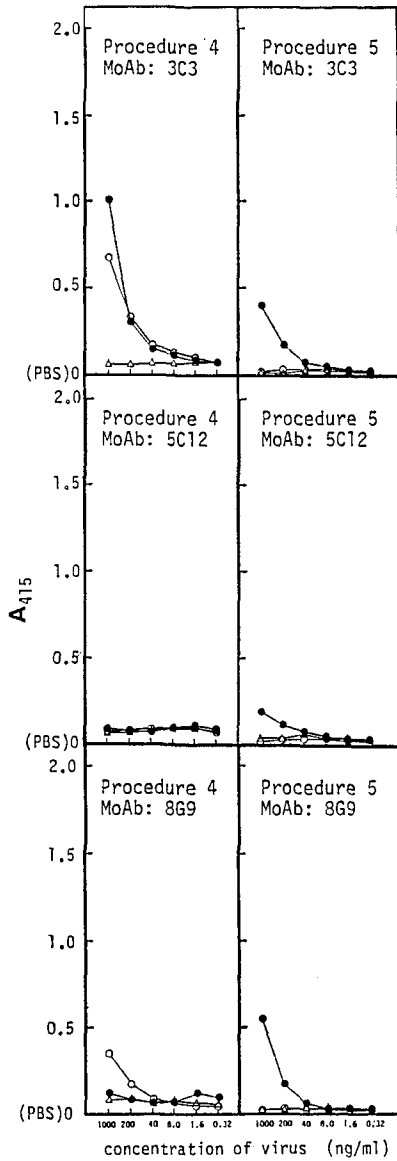


Fig. 2. Detection of the three luteoviruses by direct-ELISA using PLRV monoclonal antibodies in combination with PLRV polyclonal antibodies. Steps of ELISA procedures are shown in Table 1. The first and second antibodies were incubated for 3 hr at 37°C. The antigen of purified virus, PLRV (●—●), BWYV (○—○) and BYDV (△—△) were incubated for overnight at 6°C. Hydrolysis time was 1 hr at 37°C.

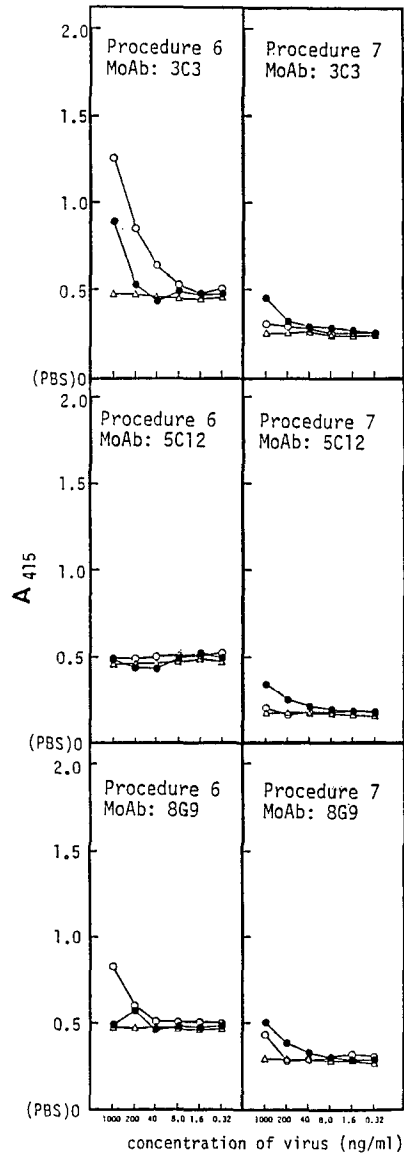


Fig. 3. Detection of the three luteoviruses by direct-ELISA using PLRV monoclonal antibodies in combination with PLRV polyclonal antibodies. Steps of ELISA procedures are shown in Table 1. The first and second antibodies were incubated for 3 hr at 37°C. The antigen of purified virus, PLRV (●—●), BWYV (○—○) and BYDV (△—△) were incubated for overnight at 6°C. Hydrolysis time was 1 hr at 37°C.

reacted slightly with BYDV in AAD- and AAI-ELISA and did not in DAS-ELISA. On the other hand, 5C12 reacted equally with each of the three viruses in the three ELISA procedures. 8G9 reacted with heterologous BWYV higher than homologous PLRV.

In general, 3C3 differentiates PLRV and BWYV from BYDV, and 8G9 almost does BWYV from PLRV and BYDV in DAS-ELISA.

**ELISA using monoclonal antibodies in combination with polyclonal antibodies.** Procedure of DAS-ELISA using MoAbs in combination with PoAbs are shown in Fig. 2. The reactions of ELISA using MoAbs in combination with PoAbs were generally very weak and its characteristics were distinct from previous procedures. When 3C3 and 8G9 were used for enzyme conjugated antibody on procedure 4, the reactivity of the three viruses was similar to DAS-ELISA (procedure 2) using only MoAbs. However, when 5C12 was used for the second-stage antibody, no reaction was observed on procedure 4. When MoAbs were used for the first antibody on procedure 5, only homologous reaction was detected.

Indirect-ELISA using MoAbs in combination with PoAbs are shown in Fig. 3. The reaction of procedures 6 and 7 are almost identical with that of direct-ELISA on procedure 4 and 5, respectively.

### Discussion

Seven MoAbs against PLRV were obtained. Comparative studies of direct- and indirect-ELISA using three of these MoAbs were presented.

Al MOUDALLAL *et al.*<sup>v</sup> compared the sensitivity of MoAbs against tobacco mosaic virus in detecting the binding activities among seven different procedures, and found that indirect-ELISA possessed the highest sensitivities. Their procedures are; 1. PoAb (chicken anti-TMV), antigen (TMV), MoAb (mouse anti-TMV), PoAb (rabbit anti-mouse Ig), PoAb (goat anti-rabbit Ig enzyme conjugate), 2: PoAb (sheep anti-mouse Ig), MoAb (mouse anti-TMV), antigen (TMV), PoAb (chicken anti-TMV), PoAb (rabbit anti-chicken between indirect- and DAS-ELISA using MoAbs. Furthermore, HUSS *et al.*<sup>o</sup> indicated that the enzyme conjugation on a MoAb against grapevine fanleaf virus destroyed all the binding activities in DAS-ELISA. However, the reaction of 5C12 MoAb against PLRV on DAS-ELISA obtained in our experiment showed high binding activity, which is as sensitive as that of PLRV PoAb on DAS-ELISA (Fig. 1, Fig. 4).

On the other hand, as shown in Fig. 2, ELISA values varied according to the combination of MoAbs and PoAbs.

It was shown that MoAbs raised against TMV did not reacted with



TMV in AAI-ELISA<sup>10</sup>. On the contrary, three MoAbs, 3C3, 5C12 and 8G9 showed high binding activity in AAI-ELISA. Inversely, MoAbs used in combination with PoAb reacted less on both procedure 4, 5 (DAS-ELISA) and procedure 6, 7 than procedure 3 (AAI-ELISA) (Fig. 5). These result lead us a conclusion that the difference in reactivities of ELISA procedures between by Al MOUDALLAL *et al.*<sup>10</sup> and by us were due to the difference in the methods of screening cell fusion products in detecting virus binding activities.

MASSALSKI and HARRISON<sup>10</sup> investigated common epitope among PLRV, BWYV and BYDV using MoAbs against British isolate of PLRV, but could not find the MoAb which reacted with BWYV and BYDV. The ELISA procedure that they used for investigation was procedure 6

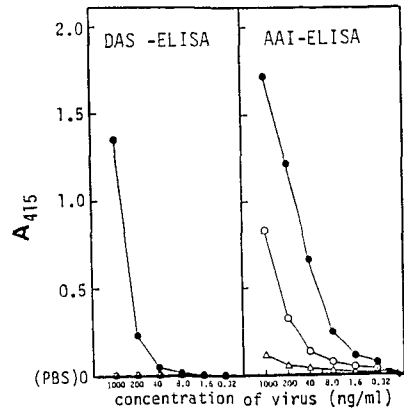


Fig. 4. Detection of the three reoviruses by DAS- and AAI-ELISA using PLRV polyclonal antibodies. The first and second antibodies were incubated for 3 hr at 37°C. The antigen of purified virus, PLRV (●-●), BWYV (○-○) and BYDV (△-△) were incubated for overnight at 6°C. Hydrolysis time was 1 hr at 37°C.

| Mono-clones | Anti-gens | procedure 1 | procedure 2 | procedure 3 | procedure 4 | procedure 5 | procedure 6 | procedure 7 |
|-------------|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|             |           | AAD         | DAS         | AAI         |             |             |             |             |
| 3C3         | PLRV      | ++          | ++          | +++         | ++          | ±           | ±           | ±           |
|             | BWYV      | ++          | +           | +++         | +           | -           | -           | -           |
|             | BYDV      | ±           | -           | ±           | -           | -           | -           | -           |
| 5C12        | PLRV      | ++          | +++         | +           | -           | ±           | -           | ±           |
|             | BWYV      | ++          | +++         | ++          | -           | -           | -           | -           |
|             | BYDV      | ++          | ++          | +           | -           | -           | -           | -           |
| 8G9         | PLRV      | ±           | ±           | ±           | -           | +           | -           | ±           |
|             | BWYV      | ++          | +           | +           | ±           | -           | ±           | ±           |
|             | BYDV      | -           | -           | ±           | -           | -           | ±           | -           |
| Poly-clone  | PLRV      | ±           | ++          | +++         |             |             |             |             |
|             | BWYV      | ±           | -           | +           |             |             |             |             |
|             | BYDV      | ±           | -           | ±           |             |             |             |             |

Fig. 5. Comparison of three monoclonal antibodies of seven different ELISA procedures. ELISA procedures detailed to Table 1. Plus (+) and minus (-) were ranked such as (-); A<sub>415</sub>=0.0, (+); A<sub>415</sub>=0.0-0.5, (±); A<sub>415</sub>=0.5-1.0, (++) ; A<sub>415</sub>=1.0-1.5, (+++) ; A<sub>415</sub>=1.5-2.0. Polyclonal antibody (a) was used in place of first and second monoclonal antibodies.

(Table 1), in which homologous PoAb was used as the first-stage (coating) antibody. Our PLRV MoAb reacted strongly with BWYV and BYDV on procedure 1, 2 and 3 (Fig. 5), in which MoAb was only used. However, on the ELISA procedure 5 and 7, in which homologous PoAb was used as the second stage antibody, three PLRV MoAbs almost reacted only with PLRV. These results suggested that MoAb in combination with PoAb reacted almost specifically with homologous virus.

Based on these results, we conclude that MoAbs are applicable to DAS-ELISA, and found that common epitopes were existed among PLRV, BWYV and BYDV.

### Summary

Seven different ELISA procedures were employed for detecting binding activities of three monoclonal antibodies (MoAbs : 3C3, 5C12, 8G9) against potato leafroll virus (PLRV).

When MoAb 3C3 was used, antigen adsorption indirect-ELISA (AAI-ELISA) showed the highest sensitivity. On the other hand, double antibody sandwich ELISA (DAS-ELISA) was the most sensitive procedure for 5C12. The results indicate that the sensitivity of ELISA is based on characteristics of MoAb. However, DAS-ELISA is applicable for detecting the viruses using all three MoAbs.

When MoAb was used as second-stage antibody in combination with polyclonal antibodies (PoAbs), detection sensitivity drastically decreased and only homologous PLRV was reacted weakly. When MoAb was used for both stages, three MoAbs reacted strongly with PLRV, beet western yellows virus (BWYV) and barley yellow dwarf virus (BYDV) and there exist common epitopes among these three luteoviruses.

### Acknowledgments

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