Monoclonal Antibodies against Plant Viruses

— Procedure and some applications —

By YUZO NOZU

Department of Molecular Biology, National Institute of Agrobiological Resources
(Yatabe, Ibaraki, 305 Japan)

Introduction

It has been well established that monoclonal antibodies, since their initial description[8], play an important role on biological research. This technique has also been applied to studies on plant virus[2,6] and this aspect was reviewed[16]. The basic characteristics of monoclonal antibodies are that (1) the antibody specifically reactive with one epitope can be obtained, (2) a hybridoma clone, once established, can be cultured indefinitely and also can be stocked indefinitely in nitrogen liquid, and (3) a hybridoma can induce ascitic fluid containing a large amount of antibodies in an injected mouse.

This paper summarized the hybridoma cell lines which have been established in our laboratory.

Procedure for production of monoclonal antibodies

For the production of cell clones secreting antibodies, spleen cells of mice immunized with antigen i.e., plant virus to be investigated, are fused with mouse myeloma cells. Some procedures for cell fusion have been reported[3]. Described below is the one carried out in our laboratory.

Purified plant virus particles (ca. 20–100 μg per mouse) suspended in an appropriate buffer were mixed with an equal volume of Freund's complete adjuvant, and injected into peritoneal cavity of 8–10 weeks old BALB/c mice. Usually 4–5 mice were immunized. Four weeks later, the mice were boosted intravenously with the same amount of virus particles as the first injection. Three days later, the serum titer of each mouse may be checked with usual methods, and then from the mouse which showed the highest value of the serum titer the spleen was removed and cut into 10 pieces and placed on a 60–100 mesh metal sieve. The resulting cells were suspended in serum-free culture medium (RPMI). The myeloma cell line commonly used in our laboratory is P3-X63-Ag8-U1 (P3U1). The cells were maintained and were grown in the RPMI medium (consisted of RPMI-1640 solution, sodium pyruvate, Kanamycin, Penicillin-Streptomycin and Fungizone), plus 15% FCS (fetal calf serum, Gibco or Boehringer). The spleen cells (5×10^7) and myeloma cells (5×10^6) were mixed and washed with the RPMI medium. After removing the medium completely, the mixed cells were fused by gently adding 1 m*l* of 50% polyethylene glycol (Merck 4,000 GK)[3] dissolved in the RPMI medium containing 15% of dimethylsulfoxide which was prewarmed at 37°C. After 30 sec, with gently rolling the tube, 1 m*l* of the RPMI medium was added every 30 sec for 5 min. Then, by adding 1 m*l* of FCS, the fusion was stopped. The cells were pelleted and resuspended in 40 m*l* of HAT selective medium which consisted of the RPMI medium plus 15% FCS and hypoxanthine-aminopterine-thymidine solution (Flow Laboratory, Inc. U.S.A.), and were distributed by 0.1 m*l* amount to the wells of 96-well microculture plates containing 1–2 days old peritoneal macrophage (4,000 cells/0.1 m*l* HAT selective medium). The plates were incubated at 37°C in 5% humidified CO_2. The hybridoma cells were fed every 2–3 days by replacing half of the medium with fresh HAT selective medium. About 50–70% of wells were observed to contain growing cells within 2 weeks. When the wells contain enough cells and the color of the medium becomes yellowish, an aliquot (0.1 m*l*) of the culture medium is tested for the anti-

bodies to the desirable antigenic determinant. Indirect enzyme-linked immunosorbent assay (ELISA) of Clerk and Adams[1] is commonly used to screen for positive hybridoma cultures. Recently it is found that the avidin-biotin system available from Vector Laboratories, USA (Vectostain TM ABC kit) which consisted of biotin labeled antibody to mouse immuno-globulin and avidine-biotinylated peroxidase is the more sensitive method. Positive wells are detected visibly as blue color. The hybridoma cells thus selected were then twice recloned to obtain stable cell lines by limiting dilution cloning with the presence of peritoneal macrophage of mouse as feeder cells. The establised cell lines can be stocked by freezing at the cell density of 3×10^6/m*l* in 1 m*l* of the RPMI medium plus 20% FCS and 10% dimethylsulfoxide at −70°C and then storing in liquid nitrogen. In order to obtain large quantities of monoclonal antibodies, the hybridoma cells were grown in vivo as tumors in a mouse. By injecting 10^6–10^7 hybridoma cells into peritoneal cavity of Pristan-primed mice, 5–20 mg of antibodies/m*l* of ascitic fluid were obtained 5 to 7 days later.

Application of monoclonal antibodies

1) Satsuma dwarf virus (SDV)[11]

Monoclonal antibodies, which permit rapid and accurate diagnosis of viral infection, may be useful

Table 1. The titers of culture media of each hybridoma cell against SDV and CiMV by the interfacial ring test

Cell lines	Antigen			
	SDV ×5	CiMV ×5	SDV ×2[a]	CiMV ×2[a]
1F6. F11. H 9	+	−	+	−
1F6. F11. C 10	+	−	+	−
1F6. F11. G 9	+	−	+	−
7F9. A10. A 1	+	+	n.t	n.t[b]
7F9. A10. E 2	+	+	n.t	n.t
7F9. A10. B 3	+	+	n.t	n.t
7F9. A10. B 6	+	+	n.t	n.t
7F9. E10. D 9	+	+	+	+
7F9. E10. C 10	+	+	n.t	n.t
7F9. E10. E 12	+	−	+	+

a) Dilution of culture media
b) Not tested

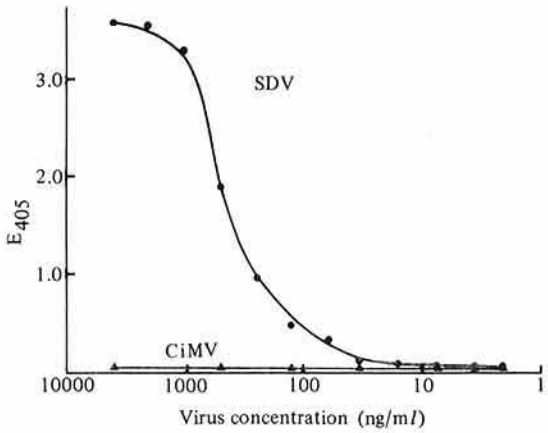


Fig. 1. ELISA tests on SDV and CiMV with monoclonal antibodies
Gamma-globulin was prepared from the ascitic fluid obtained by injecting the cell line 1F6.F11.H 9 into a mouse.

especially in fruit trees with great advantage, because the large scale diagnoses of young trees are required. Two citrus viruses, SDV[18] and citrus mosaic virus (CiMV)[20], were found to be antigenically closely related. In order to distinguish these viruses, we started to produce the monoclonal antibodies to SDV. Purified virus particles[18] (ca. 100 μg per mouse) were used as an antigen. Two hybridoma cell lines, 1F6 and 7F9, were found to secrete the anti-SDV antibodies and then twice recloned. As shown in Table 1, one cell line, 1F6, is reactive only with SDV, and the other, 7F9, both with SDV and CiMV. The specificity of the cell line, 1F6, was further proved using ELISA developed by Usugi[19]. As shown in Fig. 1, the monoclonal antibody does distinguish clearly between SDV and CiMV. The monoclonal antibody specific for CiMV is expected to be produced from the mouse immunized with CiMV as well.

2) Rice stripe virus (RSV)[14]

RSV causes one of the most severe diseases of rice in Japan. For the forecasting of the disease, the virus antigen in viruliferous insects has to be detected by the immunological method. Therefore, a large amount of antibodies must be supplied.

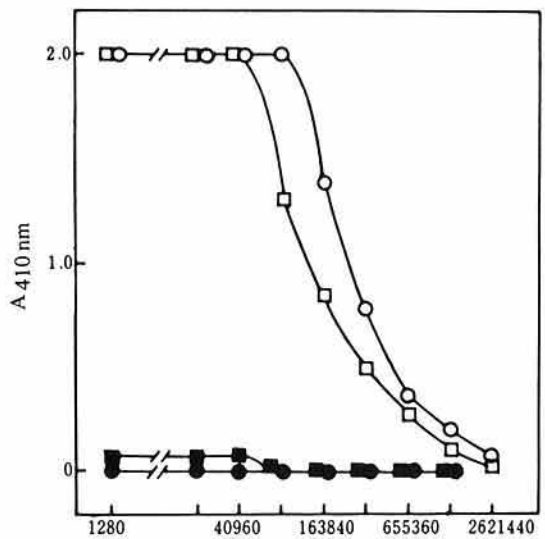
Purified virus particles[7] (ca. 20 μg per mouse) were used as an antigen. Indirect ELISA was used

to screen antibody activity. Several cell lines were obtained and recloned. The yield and titer of the ascitic fluid were determined. As shown in Table 2, 14 m*l* to 22.45 m*l* of the ascitic fluid was obtained from each mouse, and the titer was 800 to 1,600 with precipitin ring interface test. ELISA tests using avidin-biotin system showed the titer of 10^6 to 10^8. As shown in Fig. 2, the virus antigen in the field plants was detected by ELISA using the monoclonal antibodies. The characteristics as well as the amount and titer of these monoclonal antibodies are comparable to the rabbit antibody. Therefore, rabbit antisera may be replaced by monoclonal antibodies.

Table 2. Changes in the yield and titer of ascitic fluid during serial passages of hybridoma cells

Mouse no.		Number of passages		
		1	2	3
1	Amount (m*l*)	22.5	10.3	6.5
	Titer[a)]	1600	800	400
2	Amount	17.3	14.5	5.5
	Titer	800	800	400
3	Amount	14	4.6	3.5
	Titer	800	800	400

a) Precipitin ring interface test



○ Infected sap detected using monoclonal antibody
● Healthy sap detected using monoclonal antibody
□ Infected sap detected using antiserum produced in rabbit
■ Healthy sap detected using antiserum produced in rabbit

Fig. 2. Enzyme-linked immunosorbent assays for detecting RSV in sap of infected leaves

3) Rice gall dwarf virus (RGDV)[10)]

RGDV is a recently described virus of rice. The genome consists of 12 segments of double stranded RNA, and by polyacrylamide gel electrophoresis several components of proteins are found[12)]. The monoclonal antibodies against intact particles of RGDV were produced (manuscript in preparation). One of the purposes is to obtain the antibody specific for each component protein. Purified RGDV particles[13)] (ca. 20 μg per each mouse) were used as an antigen. About 30 hybridoma clones which showed positive antibody activity to RGDV particles were obtained by screening test with ELISA. Several cell lines were established. Fig. 3 shows the titer of ascitic fluid as well as culture medium. The titer of the ascitic fluid was 10^5 to 10^6 by ELISA test. Specificity for monoclonal antibodies was tested by immunoblotting method which was originally developed by Towbin et al[17)]. Component proteins were resolved by polyacrylamide gel electrophoresis into seven polypeptide bands, which showed mol. wt. of 183,000 (183K), 165K, 150K, 143K, 120K, 56K, and 45K. These polypeptides were transferred from the gel without staining to the nitrocellulose membrane. The membrane is then incubated with the culture medium. Which polypeptides this antibody is bound to is visualized by developing color with biotin-avidin conjugate system of ABC kit. Plate 1 showed that the monoclonal antibodies from two cell lines recognized 45K protein, which is located on the surface of a virus particle, although the serum obtained from the immunized mouse recognized all of proteins. No cell lines secreting monoclonal antibodies which recognized other polypeptides than 45K were obtained in our established cell lines. This may be due to the screening method, because cell intact virus particles were bound to the plates in the ELISA.

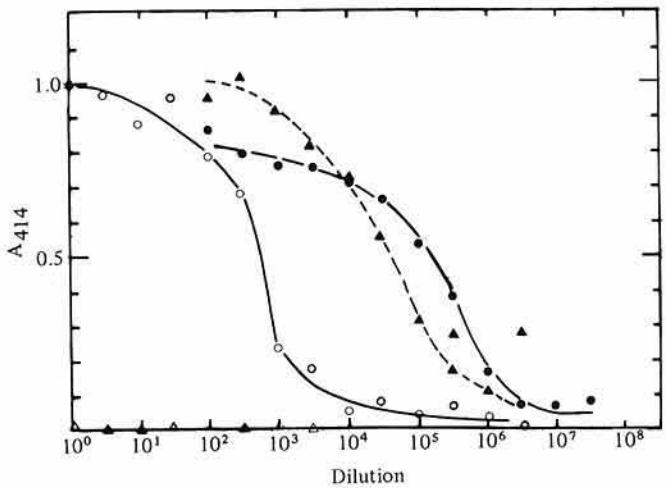


Fig. 3. The titer of antibodies to RGDV particles by ELISA

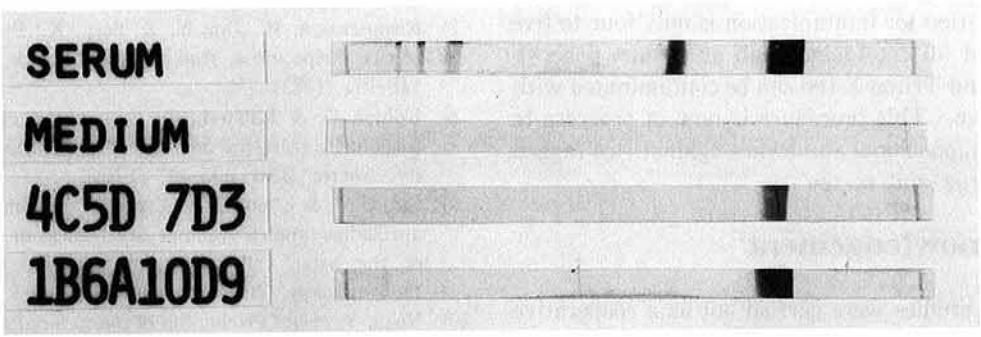


Plate 1. The component proteins of RGDV detected by the immunoblotting
The proteins were migrated from left to right by SDS-polyacrylamide gel electrophoresis.

4) Cucumber mosaic virus (CMV)[4]

CMV is widely distributed and is one of the commonest viruses in cultivated plants. Many strains were isolated and purified. Of these we noted two strains, CMV-P and CMV-Y[5]. We produced the monoclonal antibodies to CMV-P, and tested for the specificity between two strains (manuscript in preparation). Hybridoma cells were screened by the indirect ELISA system, where CMV-P and CMV-Y were coated separately on plastic plates. Of the total 23 clones, 15 clones were specific for CMV-P which was used as the immunogen, and 8 clones were reactive with both CMV-P and CMV-Y. These antibodies were found not to be reactive with peanut stunt virus and chrysanthemum mild mottle virus. We have established the cell lines and will apply them to the analysis of antigenesity of CMV group and also diagnosis studies.

Concluding remarks

The fundamental properties of monoclonal anti-

bodies will be further utilized in the study of plant virus, and also be adopted to mass production of antibody for a standardized test in epidemiological investigations. The another advantage of monoclonal antibodies is that a crude antigen or partially purified virus can be used as an antigen. If there are methods for screening an aimed antigen, the monoclonal antibodies could be separated from those secreting host specific antibodies. Hybridoma clones secreting specific monoclonal antibodies against a mycoplasmalike organism, aster yellow agent, were produced[9].

Recently another improvement for procedure was reported. Instead of immunization by injecting an antigen to a mouse, immunizing in vitro can stimulate proliferation of specific B-cell lymphocytes (see review[15]). Spleen cells of a mouse were mixed with an antigen in the presence of an adjuvant peptide. Four to five days later, the cells were fused with myeloma cells, following the usual procedures. The method has several advantages over the ordinary method. They are (1) a comparatively small amount of antigen is required, (2) the period for immunization is only four to five days, and (3) detergents such as sodium dodecylsulfate and Triton X-100 can be contaminated with an antigen. This procedure is now in progress to produce monoclonal antobodies against rice ragged stunt virus and so on.

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