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Spurious cross-reactions between plant viruses and monoclonal antibodies can be overcome by saturating ELISA plates with milk proteins

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Summary. It has been claimed recently [Dietzgen (1986) Arch Virol 91: 163–173] that a series of monoclonal antibodies (Mabs) produced against the nepovirus, arabis mosaic virus (ArMV) cross-reacted with the tobamovirus, tobacco mosaic virus (TMV). In the present report, this alleged cross-reactivity was re-examined by two ELISA procedures using Mabs produced against each of the two viruses. It was found that when highly concentrated preparations of Mabs were used, all antibodies reacted in a nonspecific manner with several plant viruses. However, when defatted milk instead of bovine serum albumin was used both as blocking agent and as diluent for the Mabs, the spurious cross-reactions between unrelated viruses were abolished. The use of milk as blocking agent did not prevent the detection of genuine cross-reactions between related nepoviruses.

Introduction

In a recent report describing the properties of 10 monoclonal antibodies (Mabs) raised against the nepovirus, arabis mosaic virus (ArMV), it was claimed that all the Mabs cross-reacted in enzyme-linked immunosorbent assay (ELISA) with two plant viruses belonging to unrelated groups, i.e. tobacco mosaic virus (tobamovirus group) and potato leafroll virus (luteovirus group) [9]. From an analysis of the fine specificity of these Mabs with respect to different ArMV strains and their corresponding dissociated coat proteins, it was concluded that the 10 Mabs recognized seven distinct antigenic determinants or epitopes of ArMV. This finding implied that a considerable number of different epitopes could be shared between the members of unrelated groups of plant viruses: such a possibility appears somewhat unlikely and would be at odds with the well-established lack of serological cross-reactivity between different plant virus groups [21].

Although a small number of unexpected cross-reactions between unrelated viruses have been reported previously [5], such findings seem to arise from

nonspecific interactions between immunoglobulin molecules and viral coat proteins [4, 10]. Since Mabs have been raised in our laboratories to both tobacco mosaic virus (TMV) [1, 3] and ArMV [13], we decided to reinvestigate the alleged antigenic cross-reactivity between these two viruses.

Materials and methods

Purified preparations of TMV (common strain) containing dissociated viral subunits in addition to intact virus particles were from laboratory stocks [12]. Mab 121 P which is specific for TMV protein has been described previously [3]. Five ArMV isolates (Cadman, Syrah, Tannat, 862, and Hop) and the F 13 strain of grapevine fanleaf virus (GFLV) were used as representatives of the nepovirus group [13, 14]. Mab 17 × 4 specific for ArMV (Syrah isolate) and Mab 161 P were used either as culture supernatants or as ascitic fluids. Suspension mass cultures of hybridoma were grown in 75 ml flasks using Dulbecco's modified Eagles medium supplemented with fetal calf serum. Supernatants obtained from these cultures contained 0.3 mg/ml protein. Other viruses included in the analysis were turnip yellow mosaic virus (TYMV), tomato bushy stunt virus (TBSV), and broad bean mottle virus (BBMV). The reactivity of the Mabs with the different viruses was tested by two ELISA procedures.

Procedure 1 is a double-antibody sandwhich (DAS) ELISA in which microtiter plates (Nunc F 96) were first coated with yolk immunoglobulins specific for different viruses [22]. After incubation with viral antigen and saturation of remaining sites on the plastic with a blocking agent such as bovine serum albumin (BSA) or defatted milk [23], the plates were incubated successively with Mabs, anti-mouse rabbit globulins (1/5000 dilution), anti-rabbit goat globulins conjugated with alkaline phosphatase (1/2000 dilution) and the substrate p-nitrophenylphosphate (1 mg/ml). After hydrolysis, absorbance at 405 nm was read using a Titertek Multiskan MC photometer (Flow Laboaratories). This ELISA procedure 1 corresponds to the procedure 4 described by Al Moudallal et al. [2].

Procedure 2 is an antigen-coated plastic (ACP) type of ELISA in which microtiter plates are first coated by 18 h incubation at 4 °C with purified preparations (1 μg/ml) of the different viruses in 0.05 M carbonate buffer, pH 9.6. Subsequent steps of the assay are as in ELISA procedure 1 described above.

Results

Initial experiments were carried out using the conditions described by Dietzgen [7, 9]. Anti-ArMV Mab 17 × 4 was allowed to react in DAS-ELISA (procedure 1) with ArMV or TMV. The Mab was used as a culture supernatant concentrated to the level of 1 mg/ml protein [8, 9]. Under these conditions, the following absorbances were observed after 30 min substrate incubation: > 2.0 with ArMV, 1.45 with TMV, 1.23 with BSA used as blocking agent and 0.3 with buffer alone. Since these results indicated that the Mab reacted strongly in a nonspecific manner with both TMV and BSA, ACP-ELISA experiments (procedure 2) were carried out to establish if the use of defatted milk as blocking agent decreased background values. After incubation with antigen, plates were saturated either with a 1% preparation of powdered, defatted milk in phosphate buffered saline, pH 7.3, containing 0.05% Tween 20 (PBS-T) or with 1% BSA in PBS-T. Mab 17 × 4 was used as ascitic fluid diluted 1/40 either in PBS-T, in PBS-T containing 1% BSA or in PBS-T containing 1% milk.

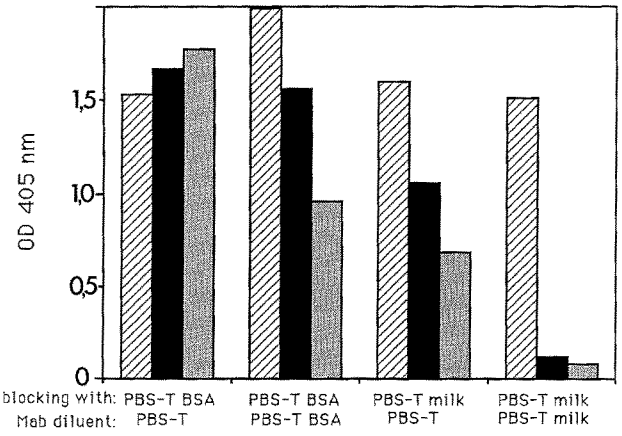

Fig. 1. Comparative effectiveness of BSA and milk proteins as blocking agents in ELISA procedure 2. Plates were coated with purified preparations (1 µg/ml) of ArMV (▨), TMV (■) or were left uncoated (▩). After blocking for 1 h at 37 °C with PBS-T-BSA or PBS-T-milk, anti-ArMV Mab 17 × 4 (ascitic fluid diluted either in PBS-T, PBS-T-BSA, or PBS-T-milk) was incubated for 2 h at 37 °C. After rinsing with PBS-T, plates were incubated successively with rabbit anti-mouse globulins, goat anti-rabbit globulins conjugated to alkaline phosphatase and substrate. Hydrolysis time was 20 min

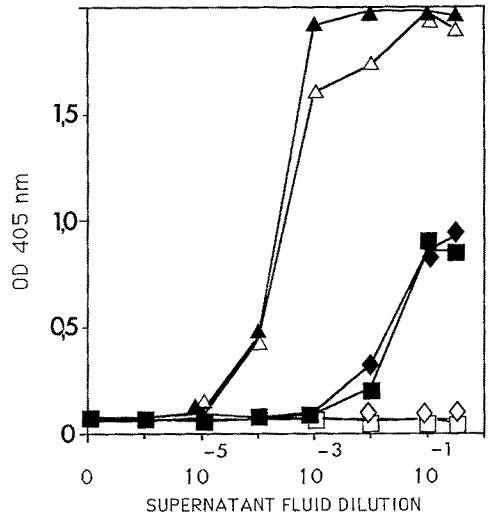

Fig. 2. Influence of antibody concentration on binding specificity of Mab 17 × 4 in ELISA procedure 1. Plates pre-coated with homologous chicken immunoglobulins were incubated with ArMV (△, ▲), TMV (□, ■), or buffer (◇, ◆). The plates were then saturated with PBS-T-BSA and incubated with Mab 17 × 4 diluted in PBS-T (▲, ■, ◆), or alternatively saturated with PBS-T-milk and incubated with Mab 17 × 4 diluted in PBS-T-milk T (△, □, ◇). Subsequent steps of the assay were as in Fig. 1

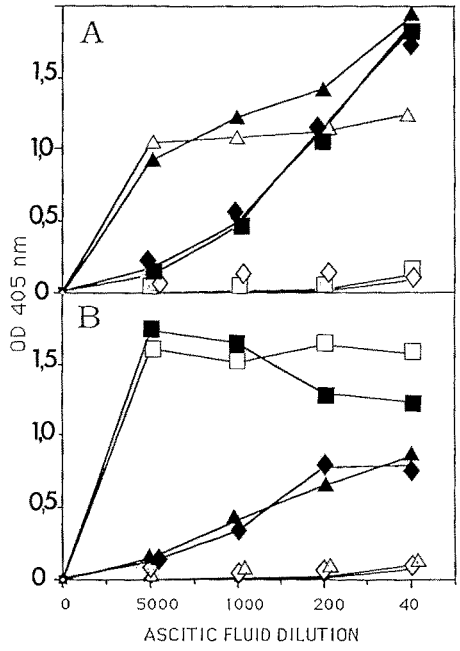

Fig. 3. Spurious cross-reactions between ArMV (△, ▲) and TMV (□, ■) demonstrated with both anti-ArMV Mab 17 × 4 (**A**) and anti-TMV Mab 121 P (**B**) in ELISA procedure 2. ◇, ◆ Buffer controls. ▲, ■, ◆ Use of PBS-T-BSA, as blocking agent and diluent. △, □, ◇ Use of PBS-T-milk. Assay conditions were as in Fig. 1

The results shown in Fig. 1 indicate that the saturation step with BSA, on its own, was totally ineffective, but that the combined use of milk as blocking agent and diluent for the Mab totally abolished nonspecific binding.

When the antibody activity present in a hybridoma supernatant (Mab 17 × 4) was titrated over the range 10^{-1} to 10^{-6} by DAS-ELISA procedure 1, the results shown in Fig. 2 were obtained. At a dilution of 10^{-1} and 10^{-2}, TMV reacted in a nonspecific manner when BSA was used as blocking agent. However, when milk was used as blocking agent and diluent, no spurious reactions were observed.

It was also of interest to demonstrate the spurious nature of the cross-reactivity between TMV and ArMV by ELISA procedure 2 using Mabs specific for either of the two viruses. In this procedure, dissociated viral subunits instead of intact viral proteins are preferentially adsorbed to the solid-phase. This was shown in a recent study combining immunoelectron microscopy and ELISA which demonstrated that when a solid-phase is coated with virus at pH 9.6, it is mainly viral subunits released from virions under the influence of the alkaline pH that become adsorbed [12]. Since the potential for observing a cross-reactivity between two viruses is enhanced when dissociated viral subunits instead of intact virions are compared [16], the alleged cross-reactivity between ArMV and TMV was also tested in reciprocal tests using ACP-ELISA. The

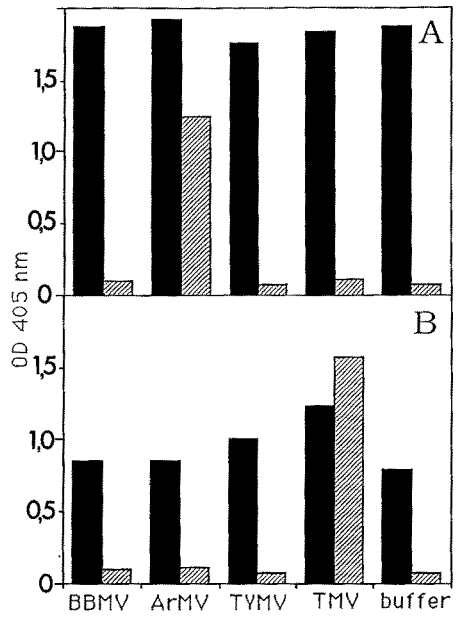

Fig. 4. Spurious cross-reactions between four plant viruses demonstrated with **A** Mab 17 × 4, **B** Mab 121 P. ■ Use of PBS-T-BSA as blocking agent and diluent. ▨ Use of PBS-T-milk. Assay conditions of ELISA procedure 2 were as in Fig. 1

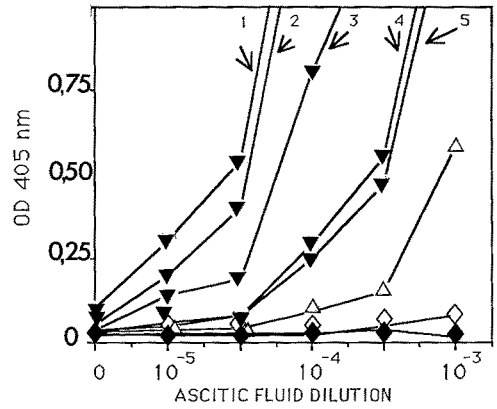

Fig. 5. Cross-reactivity between various isolates of ArMV (▼,*1–5*) and the related nepovirus GFLV (△) demonstrated with Mab 17 × 4 in ELISA procedure 2. Assay conditions were as in Fig. 1. ◇ TBSV. ◆ Buffer control

results obtained with Mab 121 P (anti-TMV protein) and Mab 17 × 4 (anti-ArMV) using ascitic fluids diluted in the range 1/40 to 1/5000 again demonstrated the spurious nature of the cross-reactivity observed with these two Mabs (Fig. 3 A and B). The level of absorbance reached with the heterologous virus was the same as with BSA (i.e., in the absence of viral antigen), while the presence of milk totally abolished the alleged cross-reactivity.

When the ELISA procedure used for obtaining Fig. 3 was applied to different plant viruses using Mabs 17 × 4 and 121 P (ascitic fluids diluted 1/40), and BSA as blocking agent, spurious cross-reactions were observed in all cases (Fig. 4). On the other hand, genuine cross-reactions between related nepoviruses could be detected in the normal way when PBS-T-milk was used as blocking agent and diluent. As shown in Fig. 5, the ability of Mab 17 × 4 to cross-react with various ArMV isolates and with the related GFLV was clearly revealed in the range of ascitic fluid dilutions 10^{-3} to 10^{-5}.

Discussion

Our data indicate that when the presence of antigenic cross-reactions between plant viruses is examined using very high concentrations of Mabs, it is essential to use in the immunoassay a very efficient blocking agent such as milk. In ELISA, antigen concentrations of 1–10 μg/ml and culture supernatant concentrations of 0.1–1.0 mg protein which correspond to the conditions used by Dietzgen [7, 9] are clearly unsuitable for establishing the existence of genuine cross-reactions between antigens, at least when the usual blocking agent, BSA, is used. Spurious nonspecific reactions can usually be avoided by diluting the antibody preparation at least 10^3 fold but it is preferable in any case to saturate the plates with milk proteins. It is well-documented that electrostatic interactions between antigen and antibody can give rise to nonspecific binding, especially when the reactants are used at relatively high concentrations, for instance in precipitation tests [17, 6, 4] or when the antigen is fairly basic or contains basic domains [18, 11]. In sensitive immunoassays such as immunoblotting [15] and ELISA, high concentrations of antigen and antibody (1/10–1/100 dilutions) should be avoided. In most cases, such high reagent concentrations are superfluous anyway as the very sensitivity of these techniques allows genuine, immunologically specific reactions to be observed at low reactant concentrations. Our results clearly demonstrate that the alleged antigenic cross-reactivity between tobamo- and nepoviruses reported by Dietzgen [9] is a spurious phenomenon caused by the nonspecific reaction of monoclonal antibodies under the conditions used in the assay. Although we presented only data obtained with two Mabs, similar spurious cross-reactions have been observed when seven other Mabs (three anti-ArMV and four anti-TMV) were tested in ELISA (data not shown).

Our findings also cast doubt on the validity of the reported antigenic relationship between TMV and ribulose-1,5-bisphosphate carboxylase [11]. This unexpected cross-reaction between a plant enzyme present in all green plants [21] and TMV was revealed with anti-TMV Mab 95, apparently under conditions of high reactant concentrations. Mab 95 had been found in earlier work [7, 8] to react equally well with TMV, tomato mosaic virus and ribgrass mosaic virus and to recognize a synthetic peptide corresponding to the C-terminal residues 155–158 of TMV coat protein. The cross-reactivity between TMV and

the plant enzyme was rationalized on the basis that the sequence corresponding to the three residues 151–153 of TMV protein is also found in the plant enzyme [11]. In ribgrass mosaic virus, the valine 151 present in TMV is replaced by a proline [20] but this does not affect binding by Mab 95, indicating that the epitope that is recognized is probably closer to the C-terminal end. It seems therefore unlikely that the alleged cross-reaction with the plant enzyme can be explained by the common sequence in residues 151–153. In view of the prevalence of nonspecific reactions when high concentrations of Mabs are used in this type of assay, it will be necessary to confirm the specific nature of the alleged cross-reaction between the plant enzyme and TMV by control experiments in which milk is used as a blockling agent. Alternatively, the specificity of the reaction between the plant enzyme and TMV could be investigated by control experiments with synthetic peptides representing the common tripeptide sequence.

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References

1. Al Moudallal Z, Briand JP, Van Regenmortel MHV (1982) Monoclonal antibodies as probes of the antigenic structure of tobacco mosaic virus. EMBO J 1: 1005–1010
2. Al Moudallal Z, Altschuh D, Briand JP, Van Regenmortel MHV (1984) Comparative sensitivity of different ELISA procedures for detecting monoclonal antibodies. J Immunol Methods 58: 35–43
3. Al Moudallal Z, Briand JP, Van Regenmortel MHV (1985) A major part of the polypeptide chain of tobacco mosaic virus protein is antigenic. EMBO J 4: 1231–1235
4. Altschuh D, Van Regenmortel MHV (1983) Refutation by ELISA of the alleged antigenic relationship between an isometric and a rod-shaped plant virus. Int J Microbiol 1: 13–17
5. Bercks R, Querfurth G (1971) Serologische Beziehungen zwischen einem gestreckten (tobacco mosaic) und einem isometrischen (cocksfoot mild mosaic) Virus. Phytopathol Z 90: 223–243
6. Crowle AJ (1973) Immunodiffusion, 2nd edn. Academic Press, New York
7. Dietzgen RG (1983) Monoklonale Antikörper gegen Pflanzenviren: Herstellung, Reinigung und Charakterisierung. Doctoral dissertation, University of Tübingen, Federal Republic of Germany
8. Dietzgen RG (1986) Immunological properties and biological function of monoclonal antibodies to tobacco mosaic virus. Arch Virol 87: 73–86
9. Dietzgen RG (1986) Characterisation of antigenic structure of arabic mosaic virus with monoclonal antibodies. Arch Virol 91: 163–173
10. Dietzgen RG, Francki RIB (1987) Nonspecific binding of immunoglobulins to coat proteins of certain plant viruses in immunoblots and indirect ELISA. J Virol Methods 15: 159–164
11. Dietzgen RG, Zaitlin M (1986) Tobacco mosaic virus coat protein and the large subunit of the host protein ribulose 1,5-biphosphate carboxylase share a common antigenic determinant. Virology 155: 262–266

12. Dore I, Weiss E, Altschuh D, Van Regenmortel MHV (1988) Visualization by electron microscopy of the location of tobacco mosaic virus epitopes reacting with monoclonal antibodies in enzyme immunoassay. Virology 162: 279–289
13. Huss B (1986) Diagnostic des virus du Court-Noué de la vigne et études d'interactions entre isolats: utilisation d'anticorps monoclonaux. Doctoral dissertation, University of Orsay, France
14. Huss B, Muller S, Sommermeyer G, Walter B, Van Regenmortel MHV (1987) Grapevine fanleaf virus monoclonal antibodies: their use to distinguish different isolates. J Phytopathol 119: 358–370
15. Koenig R, Burgermeister W (1986) Applications of immuno-blotting in plant virus diagnosis. In: Jones RAC, Torrance L (eds) Developments in applied biology I. Developments and applications in virus testing. Proceedings of a conference at the University of Cambridge, April 1985. Association of Applied Biologists, Wellesbourne, UK, pp 121–137
16. Shepard JF, Secor GA, Purcifull DE (1974) Immunochemical cross-reactivity between the dissociated capsid proteins of PVY group plant viruses. Virology 58: 464–475
17. Sluyser M, Rümke Ph, Hekman A (1969) Antigenicity of histones: comparative studies on histones with very high lysine content from various sources. Immunochemistry 6: 494–498
18. Sotirov N, Johns EW (1972) Rabbit precipitin antibody to chicken erythrocyte histone F2C. J Immunol 109: 686–691
19. Stollar BD, Rezuke W (1978) Separation of anti-histone antibodies from nonimmune histone-precipitating serum proteins, predominantly α2-macroglobulin. Arch Biochem Biophys 190: 398–404
20. Van Regenmortel MHV (1981) Tobamoviruses. In: Kurstak E (ed) Handbook of plant virus infections and comparative diagnosis. Elsevier/North Holland Biochemical Press, Amsterdam, pp 541–564
21. Van Regenmortel MHV (1982) Serology and immunochemistry of plant viruses. Academic Press, New York
22. Van Regenmortel MHV, Burckard J (1980) Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA). Virology 106: 327–334
23. Vogt RF Jr, Phillips DL, Henderson LO, Whitfield W, Spierto FW (1987) Quantitative differences among various proteins as blocking agents for ELISA microtiter plates. J Immunol Methods 101: 43–50

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