# Factors Affecting Selection of Epitope Specificity of Monoclonal Antibodies to Tulip Breaking Potyvirus

H. T. Hsu, J. M. Franssen, C. T. C. van der Hulst, A. F. L. M. Derks, and R. H. Lawson

First and fifth authors: U.S. Department of Agriculture, Agricultural Research Service, Florist and Nursery Crops Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland 20705; second, third, and fourth authors: Bulb Research Center, Lisse, The Netherlands.

Cooperative investigation, U.S. Department of Agriculture, Agricultural Research Service, BARC-West, and Bulb Research Centre, Lisse.



Accepted for publication 9 May 1988 (submitted for electronic processing).

## ABSTRACT

Hsu, H. T., Franssen, J. M., van der Hulst, C. T. C., Derks, A. F. L. M., and Lawson, R. H. 1988. Factors affecting selection of epitope specificity of monoclonal antibodies to tulip breaking potyvirus. Phytopathology 78:1337-1340.

Mouse monoclonal antibodies (McAbs) for cryptotopes or neotopes of tulip breaking potyvirus (TBV) were selected on antigen-coated plates. These antigenic determinants were apparently selected because of the instability of the virus in the alkaline condition. Cryptotopes are exposed when the virus is degraded, and neotopes are created due to novel antigenic specificities under a particular environment. In double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA), lower $A_{405}$ readings were obtained with TBV McAb-coated than with TBV polyclonal antibody (PAb)-coated plates. In ELISA on antigen-coated plates, detection of purified TBV was more sensitive with enzyme-labeled McAbs than with enzyme-labeled PAbs. In plates coated with TBV-infected saps, a higher $A_{405}$ reading was obtained with enzyme-labeled PAbs than with enzyme-labeled McAbs. Tulip breaking virus McAbs could be used to differentiate strains of TBV, and some also reacted to bean yellow mosaic potyvirus. One McAb also reacted to iris severe mosaic potyvirus. None of the McAbs reacted to lily symptomless carlavirus or lily potexvirus X.

*Additional keyword*: hybridomas.

Diagnosis of tulip breaking potyvirus (TBV) infection in tulip and lily based on leaf symptoms is unreliable (2,5). Some cultivars are symptomless carriers, and healthy looking plants may act as primary sources of inoculum for widespread tulip breaking disease.

The presence of TBV in plants can be determined by inoculating sap extracts of tulips on sensitive indicator plants such as *Lilium formosanum* A. Wallace (5). The presence of the virus in leaves or bulbs also can be determined by serological tests or electron microscopy. Serological methods are, however, the most feasible means for large-scale testing.

Serodiagnosis of TBV infection depends on the availability of high-quality antisera, and rabbit polyclonal antiserum has been used for virus detection. TBV-specific antiserum has been prepared by first absorbing the virion preparation with antibodies made to host plant components and then with antibodies to other contaminating viruses (8).

Detection of TBV-infected plants might be facilitated by using monoclonal antibodies (McAbs). Antibody-secreting hybridoma cell lines were generated from mice immunized with TBV, and the antibodies were compared with TBV polyclonal antiserum (8). A preliminary report was presented (16).

## MATERIALS AND METHODS

Materials and methods used in preparation of the immune spleen cells, hybridization with myeloma cells, screening, selection, cloning, and stabilization of desirable McAb-secreting hybridomas and determination of immunoglobulin isotypes were the same as those described for barley yellow dwarf virus (BYDV) and carnation etched ring virus (CERV) (15,17).

**Antigen.** Tulip breaking virus was purified from infected tulip cultivar Texas Flame at the Bulb Research Centre in Lisse, The Netherlands (8). The purified suspension was tested by enzyme-linked immunosorbent assay (ELISA) for the presence of lily symptomless carlavirus (LSV), frozen in dry ice, and under a USDA-APHIS permit, was sent to the United States for the production of monoclonal antibodies. Upon arrival, the virus was stored at −70 C until use. After extensive dialysis against phosphate-buffered saline (PBS; 0.02M phosphate, 0.15 M NaCl, pH 7.4), the preparation was checked by ELISA once again for freedom from LSV. Purified LSV, rabbit anti-LSV immunoglobulins, and alkaline phosphatase-immunoglobulin conjugate were kindly provided by T. C. Allen (Oregon State University, Corvallis). The concentration of TBV in PBS was determined by using an extinction coefficient of 2.4 $(mg/ml)^{-1}cm^{-1}$ at 260 nm.

**Antibody production in mice.** For in vivo antibody production, BALB/c mice were pristane-primed at least 1 wk before intraperitoneal injection of $10^7$ hybridoma cells in 0.3–0.5 ml of serum-free RPMI media (15,17). Ascitic fluids were collected 2–4 wk after transplantation of tumor cells. Relative activities of monoclonal antibodies produced were compared by using indirect ELISA. Antibodies produced in ascitic fluids were also sent to The Netherlands for evaluation.

**Purification of immunoglobulins and preparation of enzyme-labeled antibodies.** Immunoglobulins (monoclonal and polyclonal) were purified by using methods described by Clark and Adams (6). A DE 23 cellulose column, instead of a DE 22 cellulose column (Whatman Ltd., Maidstone, England), was used in the final purification of immunoglobulins. The purified immunoglobulins were subdivided into 1 ml volumes at 1 mg/ml and stored at −20 C. For frequent use, they were kept at 6 C, or at −20 C in 50% glycerol in water. Sodium azide, 0.02%, was added as a preservative.

Alkaline phosphatase-labeled immunoglobulins were prepared essentially according to the method of Avrameas (3). Conjugates were stored with 0.02% sodium azide in 50% glycerol at −20 C and were used at 1:500 dilution.

**Plant material and purified viruses.** Leaves of tulip cultivar Texas Flame infected with TBV, either lyophilized or stored at −20 C, were sources of infected tissues. Virus-free tulip cultivar Halcro was also included in the test. Buffer alone was used as

controls. Sap samples were prepared by grinding leaf tissues in 5× tissue weight of 0.01 M phosphate containing 0.14 M NaCl, pH 7.4 (0.01 M PBS) with a mortar and a pestle. Sap preparations were filtered through cheesecloth before use in testing.

Two isolates of TBV were purified from Texas Flame and Jack Laan cultivars as described (8). The same procedures (8) were used for preparing lily potexvirus X (LVX) from *Lilium longiflorum* Thunb. cv. White Cup. Iris severe mosaic potyvirus (ISMV) and LSV were purified from *Crocus vernus* L. cv. Remembrance and the *Lilium* hybrid cultivar Enchantment, respectively, according to protocols previously reported for LSV (7). Bean yellow mosaic potyvirus (BYMV) was prepared from infected *Vicia faba* L. cv. Whitkiem by using methods described by Beczner et al (4).

**ELISA procedure.** Plates were coated with viral antigens in carbonate buffer or with antibodies to trap the antigens (8). Detection of the trapped virus was with alkaline phosphatase-labeled anti-virus immunoglobulins or with McAbs followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulins.

Antibody-coated plates (1 μg/ml) were incubated at 37 C for 2 hr. Plates coated with viruses (5–10 μg/ml) or saps or antibody-coated plates for subsequent antigen trapping were incubated at 6 C for 16 hr. Incubation for immunoglobulins or alkaline phosphatase-labeled immunoglobulins was at 37 C for 2 hr. After addition of substrate, plates were incubated at 37 C for 60 min. The values of $A_{405}$ were determined with a Dynatech micro ELISA autoreader MR 580 (Dynatech AG, Switzerland). Wells were washed five times with tap water between each step. Experiments were repeated at least once.

TABLE 1. Properties of monoclonal antibodies derived from BALB/c mice immunized with tulip breaking virus

| Monoclonal antibody | Cell line | Immunoglobulin class/subclass | Reciprocal titer of ascitic fluid[a] |
|---|---|---|---|
| TBV McAb 1 | TBV4C7 | IgG1 | 25,600 |
| TBV McAb 2 | TBV17F3 | IgG1 | 102,400 |
| TBV McAb 3 | TBV25A1 | IgG1 | 1,638,400 |
| TBV McAb 4 | TBV25D1 | IgG1 | 409,600 |
| TBV McAb 5 | TBV26A4 | IgG2b | 1,638,400 |
| TBV McAb 6 | TBV26B1 | IgG1 | 6,553,600 |
| TBV McAb 7 | TBV27C2C4 | ...[b] | 1,638,400 |
| TBV McAb 8 | TBV27C2E9 | ...[b] | 102,400 |
| TBV McAb 9 | TBV28D4 | IgG1 | 1,638,400 |

[a]Indirect ELISA was used to determine the antibody titer of ascitic fluid. Purified virus was coated on plates. Details of tests are given in the text.
[b]Subclasses could not be determined.

TABLE 2. Serological reaction of mouse anti-TBV monoclonal antibodies to two isolates of tulip breaking virus, lily virus X, lily symptomless virus, bean yellow mosaic virus, and iris severe mosaic virus in indirect ELISA[a]

| Monoclonal antibody | TBV | | | | | |
|---|---|---|---|---|---|---|
| | Texas Flame | Jack Laan | LVX | LSV | BYMV | ISMV |
| TBV McAb 1 | + | + | − | − | + | − |
| TBV McAb 2 | + | − | − | − | − | − |
| TBV McAb 3 | + | + | − | − | + | − |
| TBV McAb 4 | + | + | − | − | + | + |
| TBV McAb 5 | + | + | − | − | − | − |
| TBV McAb 7 | + | − | − | − | + | − |
| TBV McAb 8 | + | + | − | − | + | − |

[a]ELISA plates were coated with purified virus preparations. A plus sign indicates a positive reaction; a negative sign indicates no reaction. Tulip breaking virus, TBV; lily virus X, LVX; lily symptomless virus, LSV; bean yellow mosaic virus, BYMV; and iris severe mosaic virus, ISMV.

## RESULTS

**Antibody classes and titer.** Nine different hybridoma cell lines producing antibodies, designated TBV McAb 1–9, were selected. The classes and subclasses of immunoglobulins secreted and the titer of antibodies in ascitic fluids are presented in Table 1. Antibodies produced by TBV27C2C4 and TBV27C2E9 hybridoma cell lines did not react with any subgroup-specific antibodies tested. All other McAbs tested were IgG immunoglobulins. Hybridoma cell line TBV26A4 secreted IgG2b. The remaining hybridomas secreted IgG1 immunoglobulins. Dilution end points of the antibodies in ascitic fluids when tested by indirect ELISA with purified TBV ranged from about $2.5\times 10^4$ to $6.5\times 10^6$, depending on the cell line.

**Reactivities of McAbs.** The reactivities of McAbs to two isolates of TBV, and one each of LVX, LSV, BYMV, and ISMV were determined by indirect ELISA. All McAbs tested reacted to the Texas Flame isolate of TBV (Table 2). Tulip breaking virus McAbs 1, 3, 4, 5, and 8 reacted with the Jack Laan isolate of TBV. The TBV McAbs 1, 3, 4, 7, and 8 also reacted with BYMV, whereas TBV McAbs 2 and 5 did not react with BYMV. Only TBV McAb 4 reacted to ISMV. Neither LSV nor LVX reacted with any of the monoclonals tested.

**Detection of tulip breaking potyvirus.** The TBV McAbs 2, 3, and 5 were chosen for the preparation of conjugates because of their differences in titers, subclasses (Table 1), and serological reactivity (Table 2). Low absorbancy occurred in ELISA plates coated with McAbs compared with plates coated with rabbit polyclonal antibodies (Table 3). Sensitivity of detection further decreased when enzyme-labeled McAbs were used as detecting antibodies. Coating with a mixture of the TBV McAbs 2, 3, and 5 in several

TABLE 3. Comparison of mouse monoclonal and rabbit polyclonal antibodies in detection of tulip breaking virus in double-antibody sandwich ELISA[a]

| Coating globulins | Test samples | Alkaline phosphatase-labeled globulins | | | |
|---|---|---|---|---|---|
| | | Rabbit | TBV McAb 2 | TBV McAb 3 | TBV McAb 5 |
| Rabbit antibodies | Purified virus | 2.25 | 0.17 | 0.71 | 1.23 |
| | Infected sap | 1.37 | 0.19 | 0.82 | 0.62 |
| | Healthy sap | 0.03 | 0.01 | 0.06 | 0.03 |
| | Control (buffer) | 0.02 | 0.01 | 0.02 | 0.02 |
| TBV McAb 2 | Purified virus | 0.12 | 0.02 | 0.04 | 0.06 |
| | Infected sap | 0.11 | 0.01 | 0.02 | 0.03 |
| | Healthy sap | 0.04 | 0.03 | 0.02 | 0.03 |
| | Control (buffer) | 0.02 | 0.01 | 0.02 | 0.02 |
| TBV McAb 3 | Purified virus | 0.15 | 0.02 | 0.03 | 0.05 |
| | Infected sap | 0.13 | 0.01 | 0.02 | 0.03 |
| | Healthy sap | 0.04 | 0.01 | 0.02 | 0.03 |
| | Control (buffer) | 0.02 | 0.01 | 0.02 | 0.03 |
| TBV McAb 5 | Purified virus | 0.51 | 0.02 | 0.08 | 0.08 |
| | Infected sap | 0.19 | 0.01 | 0.02 | 0.03 |
| | Healthy sap | 0.04 | 0.01 | 0.02 | 0.02 |
| | Control (buffer) | 0.02 | 0.02 | 0.03 | 0.03 |

[a]Values represent absorbance at 405 nm. Figures were the average of two duplicates. The experiments were repeated at least once.

different ratios did not improve the trapping of virus antigens (results not shown). In plates coated with anti-TBV rabbit immunoglobulins, the highest $A_{405}$ readings were observed when enzyme-labeled rabbit polyclonal antibodies were used. The lowest $A_{405}$ values in plates coated with rabbit immunoglobulin were recorded when alkaline phosphatase-labeled TBV McAb 2 was used.

In our studies, hybridomas were selected by indirect ELISA by using plates coated with purified TBV. Similar indirect ELISA tests were conducted to compare rabbit polyclonal antiserum with McAbs for detection of TBV. ELISA plates were first coated with antigen followed by alkaline phosphatase-labeled rabbit polyclonal antibodies or alkaline phosphatase-labeled McAbs. Enzyme-labeled TBV McAbs 3 and 5 gave stronger $A_{405}$ readings than enzyme-labeled rabbit immunoglobulins when plates were coated with purified virus (Table 4). The lowest reading was obtained for enzyme-labeled TBV McAb 2 when purified TBV was used to coat the plates. On the other hand, enzyme-labeled rabbit polyclonal antibodies gave the strongest $A_{405}$ reading when sap from infected plants was used in coating the ELISA plates.

## DISCUSSION

Hybridoma technology provides an important method of producing immortal cell lines capable of secreting specific McAbs (19). It is important to recognize, however, that the ELISA procedures used in screening antibody activities in culture fluids greatly influence the selection of these antibodies. Tulip breaking virus McAbs, selected by indirect ELISA where viral antigen was used to coat the plates, were not useful in double-antibody sandwich (DAS) ELISA for TBV detection, but in indirect ELISA, some are excellent reagents for TBV detection in purified suspensions.

Some of the McAbs produced to TBV also reacted with two other potyviruses, BYMV and ISMV. In other studies with a wider range of potyviruses, it was also shown that these TBV McAbs differed in reaction with several potyviruses including BYMV, ISMV, and iris mild mosaic potyvirus (12,16). Serological relationships using polyclonal antisera have been observed among potyviruses (10,14,21). Studies with other potyviral McAbs further substantiate such relationships (18). In addition to the results from our studies, cross reactivities were also reported among potyviruses using McAbs prepared to tobacco etch potyvirus (9), bean common mosaic potyvirus (24), and lettuce mosaic potyvirus (13). Because monoclonal antibodies recognize defined epitopes, they are useful tools in analysis of taxonomic relationships within members of the potyvirus group (14).

In a study with other viruses, e.g., barley yellow dwarf luteovirus, none of the five SGV isolate-specific McAbs, which were selected by indirect ELISA with antigen-coated plates, could be used as the coating antibody in DAS ELISA in detecting SGV (20). The SGV McAbs were, nevertheless, excellent reagents in indirect ELISA (20; Rochow and Hsu, unpublished results). In contrast, the RPV and MAV McAbs of BYDV could be used in DAS ELISA. Rabbit polyclonal antibody-coated plates were used, however, during the selection of RPV or MAV antibody-secreting hybridomas (15).

TABLE 4. Comparison of alkaline phosphatase-labeled mouse monoclonal and rabbit polyclonal antibodies in detection of tulip breaking virus when antigen samples were used to coat ELISA plates[a]

| Coating samples | Alkaline phosphatase-labeled globulins | | | |
|---|---|---|---|---|
| | Rabbit antibodies | TBV McAb 2 | TBV McAb 3 | TBV McAb 5 |
| Purified virus | 1.27 | 0.55 | 2.83 | 2.63 |
| Infected sap | 0.47 | 0.05 | 0.15 | 0.11 |
| Healthy sap | 0.04 | 0.01 | 0.03 | 0.04 |
| Control buffer | 0.00 | 0.00 | 0.00 | 0.01 |

[a] Values represent absorbance at 405 nm. Figures were the average of two duplicates. The experiments were repeated at least once.

Screening hybridomas-secreting McAbs to virions that are stable under alkaline conditions can sometimes be done with antigen-coated plates. Thus, all McAbs produced to CERV have been successfully used in DAS ELISA for detection of the virus and in immune-electron microscopy for identification of the virions of CERV (17). Selection of McAbs reactive to cryptotopes of CERV, however, may require specific methods to dissociate virions.

Our studies indicate that virions of TBV may undergo structural and conformational changes when injected into animals. Moreover, the method of screening for antibody-secreting hybridomas results in selection of antibodies with certain characteristics. Applicability of the resulting McAbs for specific uses must be evaluated on the basis of the type of test employed. Monoclonal antibodies specific to only one epitope may show reactivity only under precisely defined test conditions. When we undertook this study, we neither knew nor anticipated that TBV McAbs selected by antigen-coated ELISA would result in their usages only in indirect ELISA. This specificity in applicability may not be a limitation for conventional polyclonal antibodies, as antiserum contains mixtures of diverse immunoglobulin population representing all possible epitopes of a given antigen. A proper design of screening methods is, therefore, very important when considering the use of McAbs (1,11,23).

Unless viral antigens are stable in alkaline environments, the use of virus-coated plates in screening monoclonal antibody activities may tend to select antibodies for antigenic determinants not available in virions at other pHs. Virions of BYDV were not stable in an alkaline condition (22). During the coating step, virions may undergo degradation, thus exposing cryptotopes that were apparently less accessible to antibodies interacting with intact virions of SGV (20). In addition, coating viruses in alkaline condition may also alter the antigenic structure due to conformational changes of virion proteins, thus creating neotopes not present in intact virions. A similar change may have occurred to virions of TBV during the incubation in alkaline environments. These epitopes are normally not present on the surface of intact virions, and antibodies formed to the cryptotopes or neotopes will not react with the intact virion in DAS ELISA. Where stability of viruses at alkaline pH is a problem, rabbit antisera should be used for virus trapping to select McAbs useful in DAS ELISA. With viruses such as CERV, which is relatively stable at alkaline pH, either method (antigen-coated plates or antigens trapped on rabbit antiserum-coated plates) may select McAbs suitable for use in DAS ELISA for antigen detection.

## LITERATURE CITED


1. Al Moudallal, Z., Altschuh, D., Briand, F. P., and van Regemortel, M. H. V. 1984. Comparative sensitivity of different ELISA procedures for detecting monoclonal antibodies. J. Immunol. Methods 68:35-43.
2. Asjes, C. F., and Elbertsen, M. 1982. Tulpemozaiekvirus in tulpen-De Symptomen en het Ziehzoehen. CAD voor de Bloembollenteelt/ Ministeric van Landboun en Vissery, Lisse Den Haag. 40 pp.
3. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry 6:43-52.
4. Beczner, L., Mast, D. E., and Bos, L. 1976. The relationships between pea necrosis virus and bean yellow mosaic virus. Neth. J. Plant Pathol. 82:41-50.
5. Brierley, P., and Smith, F. F. 1944. Studies on lily virus diseases: The mottle group. Phytopathology 34:718-746.
6. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
7. Derks, A. F. L. M., and Vink-van den Abeele, J. L. 1980. Purification of lily symptomless virus. Use and value of antisera against intact and pyrrolidine-degraded virus for testing lilies and tulips. Neth. J. Plant Pathol. 86:239-250.
8. Derks, A. F. L. M., Vink-van den Abeele, J. L., and van Schadewyk, A. R. 1982. Purification of tulip breaking virus and production of antisera for use in ELISA. Neth. J. Plant Pathol. 88:87-98.
9. Dougherty, W. G., Willis, L., and Johnson, R. E. 1985. Topographic analysis of tobacco etch virus capsid protein epitopes. Virology 144:66-72.
10. Edwardson, J. R. 1974. Some properties of the potato virus Y-group.

Fla. Agric. Exp. Stn. Ser. No. 4. 398 pages.
11. Gugerli, P., and Fries, P. 1983. Characterization of monoclonal antibodies to potato virus Y and their use for virus detection. J. Gen Virol. 64:2471-2477.
12. Hammond, J., Lawson, R. H., and Hsu, H. T. 1985. Use of a monoclonal antibody reactive with several potyviruses for detection and identification in combination with virus-specific antisera. (Abstr.) Phytopathology 75:1353.
13. Hill, E. L., Hill, J. H., and Durand, D. P. 1984. Production of monoclonal antibodies to viruses in the potyviruses group: Use in radio-immunoassay. J. Gen. Virol. 65:525-532.
14. Hollings, M., and Brunt, A. A. 1981. Potyvirus group. No. 245. In: Descriptions of Plant Viruses. Commonwealth Mycological Institute, Association of Applied Biology, Kew, Surrey, U.K.
15. Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. Phytopathology 74:600-605.
16. Hsu, H. T., Franssen, J. M., Hammond, J., Derks, A. F. L. N., and Lawson, R. H. 1986. Some properties of mouse monoclonal antibodies produced to tulip breaking virus. (Abstr.) Phytopathology 76:1132.
17. Hsu, H. T., and Lawson, R. H. 1985. Comparison of mouse monoclonal antibodies and polyclonal antibodies from chicken egg yolk and rabbit for assay of carnation etched ring virus. Phytopathology 75:778-783.
18. Jordan, R. L., and Hammond, J. 1986. Analysis of antigenic specificity of monoclonal antibodies to several potyviruses. (Abstr.) Phytopathology 76:1091.
19. Kohler, G., and Milstein, C. 1975. Continuous culture of fused cell secreting antibodies of predefined specificity. Nature 256:495-497.
20. Rochow, W. F., Hu, J. S., Forster, R. L., and Hsu, H. T. 1987. Parallel identification of five luteoviruses that cause barley yellow dwarf. Plant Dis. 71:272-275.
21. Shepard, J. F., Secor, G. A., and Purcifull, D. R. 1974. Immunological cross-reactivity between the dissociated capsid proteins of PVY group plant viruses. Virology 58:464-475.
22. Slack, S. A., Rochow, W. F., and Hsu, H. T. 1984. Molarity and pH effects on five barley yellow dwarf virus isolates. (Abstr.) Phytopathology 74:801.
23. Underwood, P. A., and Bean, P. A. 1985. The influence of methods of production, purification and storage of monoclonal antibodies upon their observed specificities. J. Immunol. Methods 80:189-197.
24. Wang, W.-Y., Mink, G. I., and Silbernagel, M. J. 1985. A broad spectrum monoclonal antibody prepared against bean common mosaic virus. (Abstr.) Phytopathology 75:1352.