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# Monoclonal antibodies as probes of the antigenic structure of tobacco mosaic virus

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**The antigenic structure of tobacco mosaic virus has been analysed by measuring the ability of nine monoclonal antibodies to distinguish between wild-type virus and 13 mutants showing single and double amino acid substitutions in the coat protein. Although the majority of antibodies detected those substitutions that were located at the outer surface of the virion, some of them also recognized conformation alterations induced by exchanges occurring deep inside the subunit. In the case of five mutants, the antibody reactivity was reduced compared with wild-type virus, while in the case of three others, it was significantly higher. Each monoclonal antibody possessed a unique discrimination pattern with respect to the different substitutions. The simultaneous presence of two exchanges led to the complete disappearance of any binding with six of the nine antibodies and to reduced binding with three others. The superior discriminatory capacity of monoclonal antibodies compared with polyclonal antisera was demonstrated by the fact that three exchanges not detected with antisera were found to alter the antigenicity when tested with monoclonal antibodies.**

*Key words:* monoclonal antibodies/antigenic structure/ conformational changes/tobacco mosaic virus/viral mutants

## Introduction

The monospecificity of monoclonal antibodies makes them particularly useful for elucidating the complex antigenic structure of proteins. Proteins are multideterminant antigens, and they elicit in immunized animals a variety of heterogeneous antibody families, each family being comprised of members able to recognize with various degrees of fit, one particular antigenic determinant of the protein. This double heterogeneity of polyclonal antisera raised against proteins is responsible for the fact that an unequivocal delineation of the epitopes of proteins has been difficult to achieve. As discussed elsewhere (Van Regenmortel, 1982), attempts to localize the epitopes of the two proteins, myoglobin and lysozyme, by means of polyclonal antisera, have led to considerable disagreement between several groups of workers (Atassi, 1977, 1979; Arnon, 1977; White *et al.*, 1978; Ibrahimi *et al.*, 1979, 1980; East *et al.*, 1980; Kabat, 1980). This lack of agreement may be ascribed to the uniqueness of each antiserum as well as to differences in methods of immunization and animal species used. Furthermore, antibody-antigen interaction was measured by different techniques and this may have led different workers to emphasize different sub-sets of the total antibody population. It is known, for instance, that techniques based on the use of highly diluted antiserum will preferentially measure antibodies of high affinity.

Evidently, the use of monoclonal antibodies represents an ideal way to obviate such difficulties inherent in the use of heterogeneous antibody mixtures. The molecular homogeneity of a monoclonal antibody preparation ensures that only one epitope is investigated at one time, and this allows protein determinants to be studied in a more defined and reproducible manner. Recently this approach has been used to study the epitopes of influenza virus hemagglutinin (Wiley *et al.*, 1981; Gerhard *et al.*, 1981; Lubeck and Gerhard, 1982), myoglobin (Berzofsky *et al.*, 1980, 1982; East *et al.*, 1982) and lysozyme (Metzger *et al.*, 1980; Smith-Gill *et al.*, 1982; Kobayashi *et al.*, 1982). The results obtained so far indicate that many monoclonal antibodies are incapable of binding to large peptide fragments of the antigen that are known, from tests with polyclonal antiserum, to harbour antigenic activity. This means that such monoclonal antibodies recognize what has been termed conformational (or topographic) determinants, i.e., epitopes that are defined by a particular folding of the polypeptide chain which is lost during fragmentation of the antigen. These antibodies are thus of little use for localizing epitopes by measuring the residual antigenic reactivity present on a series of peptides obtained by cleavage of the protein. For this reason, attempts to define protein epitopes by means of monoclonal antibodies usually use a panel of closely related proteins possessing a limited number of amino acid substitutions. If a particular substitution leads to a change in antibody binding, it is assumed that the residue is part of an epitope. Such an assumption may be justified if the three-dimensional structure of the molecule shows that the residue is situated at the surface (Smith-Gill *et al.*, 1982; Berzofsky *et al.*, 1982). Since substitutions occurring outside an epitope could also influence the structure of a distal epitope by long-range conformational effects, structural information on the folding of the peptide chain is required to support the possible involvement of external residues in any particular epitope. There is, in fact, good evidence that substitutions outside epitopes are able, by some kind of allosteric mechanism, to produce conformational changes that alter the antigenic reactivity of proteins (Hurrell *et al.*, 1977; Ibrahimi *et al.*, 1979).

We have examined the ability of monoclonal antibodies specific for tobacco mosaic virus (TMV) to detect antigenic changes induced by single amino acid substitutions in the coat protein of the virus. Some of the substitutions are located at the outer surface of the viral capsid while others are internal and embedded within the protein subunit.

TMV is a rod-shaped particle, 300 nm long, which consists of 2130 identical protein subunits arranged as a helix around an RNA molecule. The protein subunit contains 158 amino acid residues and its three-dimensional structure has been established by X-ray crystallography at a resolution of 0.28 nm (Bloomer *et al.*, 1978). The antigenic structure of TMV protein has been studied extensively and reviews are available (Benjamini, 1977; Van Regenmortel, 1982). By means of inhibition experiments, seven tryptic peptides of the protein subunit have been found to possess antigenic activity

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(Milton and Van Regenmortel, 1979; Altschuh and Van Regenmortel, 1982). Two of these peptides, corresponding to residues 62–68 and 142–158, harbour epitopes expressed at the outer surface of the viral capsid. Antigenic changes resulting from mutational events have also been studied. By comparing wild-type virus with mutants possessing 1–3 amino acid exchanges, it was found that substitutions occurring at residues 65, 66, 107, 136, 138, 140, 148, and 156 altered the antigenic properties of the virus, whereas substitutions at positions 20, 21, 25, 33, 46, 59, 63, 81, 97, 99, 126, and 129 did not (Sengbusch, 1965; Van Regenmortel, 1967). Some of these substitutions appeared to affect the antigenic reactivity of the virus and monomeric subunit by altering the conformation of the polypeptide chain (Milton *et al.*, 1980). In this respect, the exchange Pro 156→Leu is particularly interesting as it leads to a conformational change that allows the mutant virion to react with heterospecific (or heteroclitic) antibodies present in TMV antisera. Such heterospecific antibodies, which seem to be elicited in all animals immunized with TMV (Sengbusch and Wittmann, 1965; Van Regenmortel, 1966; Loor, 1971; Lelarge and Van Regenmortel, 1974), are unable to react with the immunogen in precipitin tests. The presence of these antibodies can be demonstrated by the fact that a TMV antiserum that has been fully absorbed with the homologous TMV (for instance by intragel absorption) is still capable of reacting with a viral mutant showing an exchange at position 156.

The present work was undertaken to establish whether the antigenic structure of TMV determined with polyclonal antisera could be confirmed and extended by the use of monoclonal hybridoma antibodies. Thirteen viral mutants showing single and double amino acid substitutions in the coat protein were compared by means of nine different monoclonal antibodies. Although the majority of antibodies detected those substitutions located at the outer surface of the virion, some of them also recognized conformational alterations induced by exchanges situated far away from the surface. These findings clearly illustrate the value, as well as the limitations, of monoclonal antibodies for elucidating the antigenic structure of proteins. Since the sites of binding of the monoclonal antibodies, as distinct from the site of mutation, are not known, the main value of this type of analysis lies in the unravelling of the exquisite specificity of virus-antibody interactions.

## Results

Nine stable hybridoma clones secreting TMV antibodies were derived from the fusion experiments. Four clones (3, 19, 22, and 28) were of the IgG2a class, two of the IgG2b class (8 and 21), two of the IgG3 class (18 and 20) and one of the IgG1 class. All of the antibodies possessed kappa light chains.

The ability of these nine monoclonal antibodies to differentiate between TMV and viral mutants possessing single and double amino acid substitutions in the coat protein was measured by an enzyme-linked immunosorbent assay (ELISA). The substitutions present in each of the mutants are listed in Table I. Mutants Ni 430, FU 27, Ni 1688, 414, Ni 116, Ni 109, and CP 415 were derived from wild-type TMV, while mutants Ni 458, Ni 1045, Ni 630, Ni 725, and Ni 1927 were derived from strain A14 which compared to wild-type TMV, has an Ile 129→Thr exchange (Wittmann-Liebold and Wittmann, 1965). The exchange at position 129 is situated in the LR helix of the virus (Bloomer *et al.*, 1978) and does not alter the immunochemical properties of the virus, as measured with antisera or monoclonal antibodies (Sengbusch, 1965; Table II).

Since the double sandwich indirect type of ELISA (Van Regenmortel and Burckard, 1980) was used in this work, it was necessary to establish that the different mutants were all able to bind in an identical manner to the coating antibody (goat anti-TMV serum) used in the test. As shown in Figure 1A in the case of three mutants, this ELISA test (using mouse anti-TMV serum as intermediary antibody and rabbit anti-mouse IgG antiserum as conjugate) did not differentiate between wild-type virus and the mutants. Similar results were obtained with all the mutants we studied.

When the mouse anti-TMV serum used in ELISA (Figure 1A) was replaced by monoclonal antibody 18 (Figure 1B) the reaction with mutant 414 was reduced while the reactions with mutants Ni 458, Ni 1927, and Ni 1688 were totally

**Table I.** Amino acid substitutions of mutants with respect to wild-type TMV

| | 1 | 20 | 58 | 59 | 63 | 65 | 66 | 97 | 107 | 129 | 140 | 156 | 158 | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TMV | S | P | V | T | P | S | D | E | T | I | N | P | T | |
| 430 | . | T | . | . | . | . | . | . | . | . | . | . | . | |
| FU 27 | . | . | A | . | . | . | . | . | . | . | . | . | . | c |
| Ni 458 | . | . | . | I | . | . | . | . | . | T | . | . | . | d |
| Ni 1045 | . | . | . | . | S | . | . | . | . | T | . | . | . | a,b,d |
| Ni 1688 | . | . | . | . | S | . | . | . | . | . | . | . | . | a,b,d |
| 414 | . | . | . | . | . | G | . | . | . | . | . | L | . | b,d |
| Ni 116 | . | . | . | . | . | . | G | . | . | . | . | . | . | c |
| Ni 109 | . | . | . | . | . | . | . | G | . | . | . | . | . | b |
| Ni 630 | . | . | . | . | . | . | . | . | M | T | . | . | . | b |
| Ni 725 | . | . | . | . | . | . | . | . | M | T | . | . | . | a,b,d |
| A 14 | . | . | . | . | . | . | . | . | . | T | . | . | . | a,b,d |
| CP 415 | . | . | . | . | . | . | . | . | . | . | K | . | . | a,b,d |
| Ni 1927 | . | . | . | . | . | . | . | . | . | T | . | L | . | b |
| | | | | | | | | | | | | | | b,d, |

a: Wittmann (1962).
b: Wittmann-Liebold and Wittmann (1965).
c: Funatsu and Fraenkel-Conrat (1964)
d: Wittmann (1964).

**Table II.** Comparative binding of monoclonal antibodies to wild-type TMV and to mutants with single amino acid substitutions in the coat protein

| Antibody clone number | 3 | 8 | 18 | 19 | 20 | 21 | 22 | 25 | 28 | Number of clones able to detect the exchanges |
|---|---|---|---|---|---|---|---|---|---|---|
| Substitution position | | | | | | | | | | |
| 20 | = | = | = | ⊖ | = | = | = | = | = | 1 |
| 58 | = | = | = | ⊖ | = | = | = | = | = | 1 |
| 59 | = | = | 0 | ⊖ | = | = | = | = | = | 2 |
| 63 | = | = | = | ⊕ | = | ⊕ | = | = | = | 2 |
| 65 | = | = | ⊖ | ⊕ | = | = | = | ⊖ | = | 3 |
| 66 | = | = | = | ⊕ | 0 | = | = | = | = | 2 |
| 97 | = | = | = | = | = | = | = | = | = | 0 |
| 107 | = | = | = | ⊖ | = | ⊖ | = | = | = | 2 |
| 129 | = | = | = | = | = | = | = | = | = | 0 |
| 140 | = | = | = | = | ⊖ | = | = | ⊖ | ⊖ | 3 |
| 156 | = | = | 0 | 0 | 0 | = | ⊖ | ⊖ | = | 5 |
| 65 + 156 | ⊖ | 0 | 0 | 0 | 0 | ⊖ | 0 | ⊖ | 0 | 9 |
| Number of single substitutions detected by each antibody | 0 | 0 | 3 | 8 | 3 | 2 | 1 | 3 | 1 | |

⊕ higher binding than with wild-type virus
= equal binding than with wild-type virus
⊖ lower binding than with wild-type virus
0 no binding at all

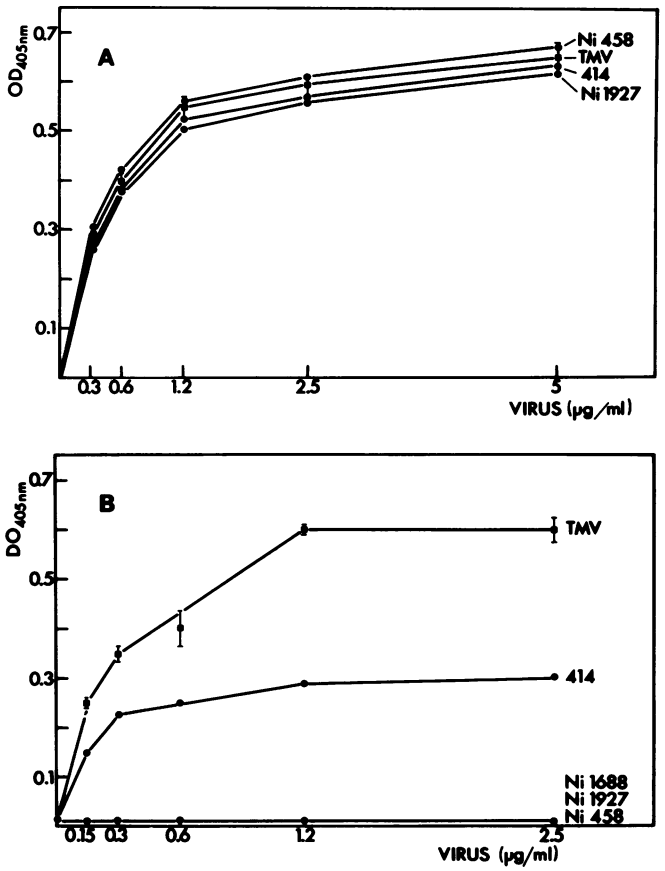


**Fig. 1.** (**A**) Detection of TMV and three mutants by indirect ELISA. Coating of wells was with 1 µg/ml goat anti-TMV globulin for 1 h. The antigens were incubated for 3 h and the intermediate antibody (mouse anti-TMV globulin diluted 1/1000) was allowed to interact for 2 h. The enzyme conjugate diluted 1/1500 was incubated for 2 h. (**B**) Comparison between four mutants and TMV by indirect ELISA. The intermediate antibody used in **A** was replaced by monoclonal antibody 18 diluted 1/60 000. All other steps of the assay were as in **A**.

abolished. The comparative binding of monoclonal antibody 19 to nine mutants is illustrated in Figure 2. In the case of five mutants, the antibody reactivity was reduced compared to wild-type virus, while in the case of three others (mutants Ni 1045, 414, and Ni 116) it was significantly higher than with TMV. Antibody 19 may thus be considered a heterospecific TMV antibody that reacts less well with the immunogen than with other related antigens. Further ELISA tests with antibodies 21 and 25 are illustrated in Figure 3; antibody 21 is also heterospecific. The results of all the comparisons between TMV and 13 mutants summarized in Table II show that each antibody possessed a unique discrimination pattern with respect to the different substitutions. Two antibodies (3 and 8) were unable to differentiate between TMV and any of the mutants with single substitutions, and showed either a reduced binding or no binding at all (8) when two substitutions (residues 63 and 156) were simultaneously present in the coat protein. The simultaneous presence of these two substitutions led to the complete disappearance of any binding with six of the nine antibodies, and to reduced binding with three others (clones 3, 21, and 25).

Attempts were made to use the monoclonal antibodies for differentiating between the dissociated coat proteins of the mutants. However, in an ELISA with TMV coat protein in the concentration range 0.1 – 2.0 µg/ml, none of the antibodies showed any measurable binding. In these experiments, the wells were coated directly with TMV protein (Altschuh and Van Regenmortel, 1982), which eliminated any potential problems with coating antibody.

## Discussion

The use of monoclonal antibodies for mapping the antigenic structure of a protein depends on the availability of a series of mutants or close variants of the molecule whose sequences are known (Berzofsky *et al.*, 1982; East *et al.*, 1982; Smith-Gill *et al.*, 1982). In the case of TMV, a large number of mutants and strains of known sequence are available (Hennig and Wittmann, 1972; Van Regenmortel, 1981) and since the three-dimensional structure of the common strain is known, it is possible to predict which amino acids are potentially capable of acting as contact residues in epitopes of the virus. The results obtained in the present study and summarized in Table II will be discussed with reference to the diagrammatic representation of the folding of the viral subunit illustrated in Figure 4.

Seven of the substitutions (at residues 58, 59, 63, 65, 66, 140, and 156) which led to altered binding by monoclonal antibodies are situated at the subunit surface that is exposed to the solvent in both capsid and protein monomer. It is therefore conceivable that all these residues participate in the formation of antigenic sites of the virus. Exchanges at positions 97 and 129 that are situated 3 – 5 nm from the capsid surface did not alter antibody binding. On the other hand, the other two substitutions that altered antibody binding (at positions 20 and 107) are located ~2 and 5 nm away from the viral surface, and they must therefore have exerted an effect through long-range conformational changes. These two clear-

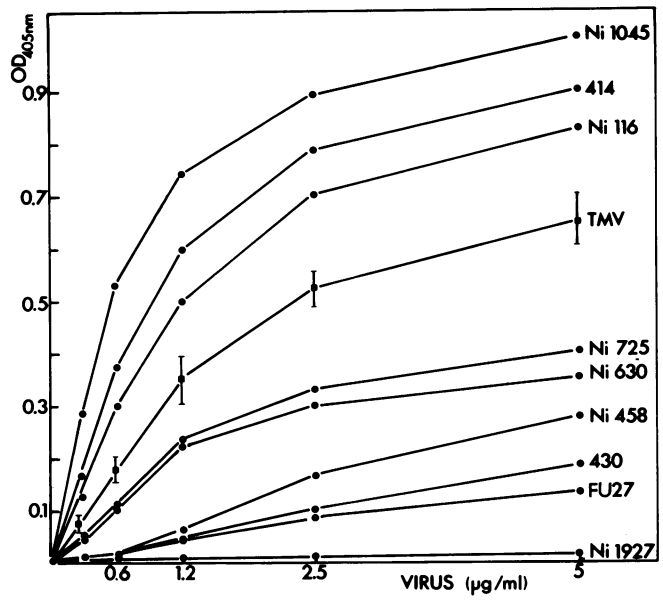


Fig. 2. Comparison between nine mutants and TMV in indirect ELISA, using monoclonal antibody 19 diluted 1/2000 as intermediary antibody.

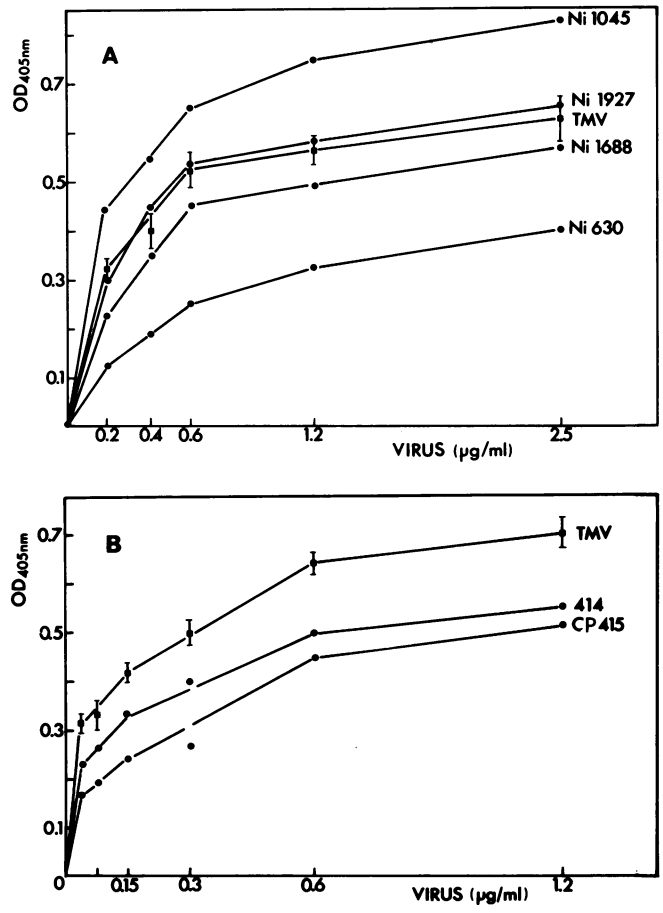


Fig. 3. (A) Comparison between four mutants and TMV in indirect ELISA, using monoclonal antibody 21 diluted 1/8000. (B) Comparison between two mutants and TMV in indirect ELISA using monoclonal antibody 25 diluted 1/2000.

cut cases of substitutions leading to antigenic changes in a distal epitope show that it is unjustified to assume that all exchanges detected by monoclonal antibodies are located in

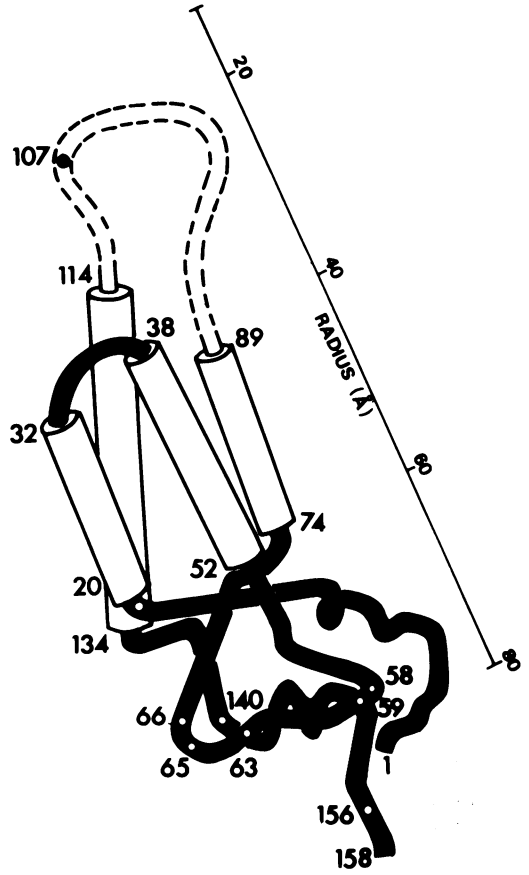


Fig. 4. Three dimensional structure of the TMV protein molecule, based on the X-ray crystallographic data of Bloomer *et al.* (1978). The radius scale starts at the centre of the hole in the assembled virion. The helices are represented as cylinders. The locations of amino acid exchanges detected with monoclonal antibodies are represented as circles (positions 20, 58, 59, 63, 65, 66, 107, 140, and 156).

contact residues at the surface of the antigen.

The capacity of different monoclonal antibodies to detect residue exchanges was found to be extremely variable (see Table II). Antibody 19 was able to recognize substitutions at eight individual locations while two others (18 and 20) recognized three different exchanges. Although some of the exchanges were recognized more frequently than others (for instance at positions 65, 140, and 156), each antibody showed a unique pattern of mutant discrimination. For instance antibody 18, which recognized three exchanges that were also detected by antibody 19, showed less binding to mutant 414, whereas antibody 19 showed increassed binding to the same mutant.

All the substitutions (at positions 65, 66, 107, 140, and 156) which in previous work had been detectable with polyclonal antisera (Sengbusch 1965; Van Regenmortel, 1967) were also recognized by some of the monoclonal antibodies. However, it should be emphasized that many individual monoclonal antibodies failed to recognize certain exchanges (e.g., at position 156) that were detected by all antisera in earlier work. Three exchanges (at positions 20, 59, and 63), which remained undetected with antisera, were found to lead to altered epitopes when tested with monoclonal antibodies. These findings illustrate the superior discriminatory capacity of some monoclonal antibodies compared to polyclonal antisera. One reason for this superiority is that, in the case of multideterminant antigens, those antibodies in an antiserum that recog-

nize an alteration in one particular epitope may be swamped by the large number of antibodies that still react in the normal way with unchanged epitopes. A second reason lies in the heterogeneity of antibodies directed to one determinant. It has been known for a long time that antibodies to a single determinant are heterogeneous with respect to their ability to cross-react with related chemical groupings (Landsteiner and Van der Scheer, 1936). Therefore, not all antibodies specific for one epitope will recognize a particular alteration equally well, and those antibodies that retain sufficient reactivity may blur the discrimination achieved by some others. A third reason for the superiority of monoclonal antibodies lies in the fact that the binding sites of antibodies are polyfunctional (Talmage, 1969; Richards and Konigsberg, 1973; Richards *et al.*, 1975; Cameron and Erlanger, 1977). The concept that an antibody combining site is multispecific has only slowly become established, presumably because it seemed to contradict the very notion of immunological specificity. It is now clear that the specificity of polyclonal antisera is due to a population phenomenon that masks the potential cross-reactivities of each individual antibody (Richards and Konigsberg, 1973; Lane and Koprowski, 1982). This additive effect, that emphasizes the common reactivity of the various antibodies, is absent with monoclonal antibodies, and it follows that heterospecific activities will be more easily detected with hybridomas (Van Regenmortel *et al.*, 1982). As shown in Figures 2 and 3A the superior reactivity of monoclonal antibodies with mutants compared with the wild-type immunogen can be clear-cut. It seems that mutations in the region 63–66 of TMV are particularly suited for demonstrating the presence of heterospecific antibodies. The small number of hybridomas analysed until now probably explains why heterospecific antibodies recognizing the exchange Pro 156→Leu (Van Regenmortel, 1982) have not yet been obtained.

It seems likely that heterospecific antibodies will increasingly be encountered in comparative studies with monoclonal antibodies. Clearly, there is no reason to expect that the clonal selection that triggers antibody production by B cell lines must necessarily correspond to a situation where the best possible fit between epitope and paratope (Jerne, 1960) occurs with the immunogen. The reactivity of the antibody may be superior with structural relatives of the immunogen, such as the mutants studied in this work.

In summary, our results confirm earlier data that demonstrated the presence of viral epitopes in the regions 62–68 (Milton and Van Regenmortel, 1979) and 153–158 (Anderer and Schlumberger, 1965; Anderer and Ströbel, 1972) of TMV protein, and show that these regions harbour topographical antigenic determinants. In addition, our results demonstrate that certain fine details of the antigenic structure, especially conformational aspects, can only be unravelled by the new hybridoma technology.

## Materials and methods

*Viruses and mutants*

TMV, common strain as well as mutants 414 and 430, were originally obtained from H. Fraenkel-Conrat (Van Regenmortel, 1967). Mutants CP 415, Ni 1688, and Ni 1927 were from H.G. Wittmann (Berlin) and mutants Ni 109, FU 27, Ni 458, Ni 1045, Ni 116, Ni 630, Ni 725, and A 14 were from K. Holmes (Heidelberg) and D. Vogel (Stuttgart).

*Immunization*

Adult male and female Balb/c mice purchased from C.E.S.A.L. (Vigneul-sous-Montmedy, France) were immunized with 100 μg TMV mixed with incomplete Freund's adjuvant. The animals received four i.m. injections at intervals of 2 weeks. A booster injection of 300 μg TMV was given i.p. 3 days before the fusion.

*Preparation of hybridomas*

Cell fusion was performed according to a modified version of the technique described by Galfre *et al.* (1977). NS 1 cells, kindly provided by C. Milstein (Cambridge) were maintained in RPMI 1640 (Bio-Mérieux, Charbonnières les Bains, France) plus 10% foetal calf serum (FCS; Flow Laboratories Ltd., Irvine, UK). Usually two spleens from immunized Balb/c mice were teased and the resultant cell suspension was passed through a stainless steel mesh. Fusion was carried out in a 5:1 ratio of splenic lymphocytes to tumor cells; the cell suspension was pelleted (400 *g*) at room temperature for 10 min. A volume of 1.5 ml of 50% (v/v) polyethylene glycol (mol. wt. 1540: Koch-Light laboratories, Colnbrook, Bucks., UK) was added. After incubation for 1 min at 37°C, RPMI-Hepes pH 7.4 was slowly added to the cell suspension according to Galfre *et al.* (1977). The cells were then diluted, washed twice with RPMI-Hepes, resuspended in RPMI 10% FCS and distributed into 96 wells microtitre plates at a concentration of $10^6$ cells/100 μl and per well. Finally, plates were placed into a 7% $CO_2$-air-incubator at 37°C. One day after fusion HAT medium was added. After 10–15 days, the supernatant from wells containing clones were tested for the presence of TMV antibodies by the ELISA test described below.

All positive clones were then recloned by using $10^7$ thymocytes/ml as feeder cells. Hybridoma cells were grown in ascites form by injecting pristan-primed Balb/c mice with 2 x $10^6$ cells. The ascitic fluid was harvested by tapping the peritoneum with a 19 gauge syringe needle and the cells and debris were removed by centrifugation at 300 *g* for 15 min. The supernatant was divided in 1 ml fractions and frozen until use.

*Detection of anti-TMV antibodies produced by hybridoma cells*

The presence of anti-TMV antibodies in supernatants of clones and in ascitic fluids was detected by ELISA as described by Van Regenmortel and Burckard (1980). Coating of wells was performed with 1 μg/ml goat anti-TMV globulin for 1 h; after rinsing TMV or mutants were incubated for 3 h; after further washing diluted supernatants or ascitic fluids were allowed to interact for 2 h. The enzyme conjugate (rabbit anti-mouse IgG diluted 1:1500) was incubated for 2 h and, after a final rinsing, the bound alkaline phosphatase conjugate was detected by adding the substrate *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8. After hydrolysis, the absorbance at 405 nm was directly read in a Dynatech micro ELISA minireader MR 590.

*Comparisons between mutants and TMV*

To normalize the comparisons, the ascitic fluid from each clone was diluted sufficiently to achieve an optical density of ~0.8 in an indirect ELISA with a TMV concentration of 2.5 μg/ml. The dilutions were as follows: clone 18, 1/60 000; clones 22 and 8, 1/12 000; clones 20, 21, and 28, 1/8000; clone 3, 1/2500; clones 19 and 25, 1/2000.

In tests with dissociated TMV protein, all the clones were used at a dilution of 1/2000.

*Hybridoma antibody class determination*

Antibody class was determined by indirect ELISA. Wells were coated for 1 h at 37°C with ascitic fluids diluted in carbonate buffer pH 9.6. Rabbit anti-mouse immunoglobulin class specific antisera (Litton Bionetics Inc., Kensington, MD) were diluted 1:1000 in phosphate buffered saline pH 7 and incubated for 2 h. The final steps consisted of incubation with goat anti-rabbit globulins conjugated with alkaline phosphatase, followed by substrate.

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