Definition of Essential Amino Acid Residues in the Recognition of a Peptide by a Mouse Monoclonal Antibody

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A mouse monoclonal antibody reacting in ELISA with a synthetic peptide representing a linear amino acid stretch of the protein antigen was tested on all overlapping 5-mer to 9-mer fragments of the peptide, as prepared by multi-pin synthesis. Analysis of the binding data suggests that several residues in the peptide might be relatively unrelevant for recognition, while few others seem to play a critical role as key residues. On the basis of such observations, we attempted to reconstruct an alternative essential epitope by introducing multiple amino acid substitutions in the 9-mer peptide exhibiting the best binding activity, and then tested its ability to be recognized by the monoclonal antibody.

Introduction

Synthetic peptides corresponding to amino acid stretches of a protein antigen may be occasionally recognized by monoclonal antibodies produced against the macromolecule. This event appears to be relatively rare, as most monoclonal antibodies seem to be essentially directed against conformational determinants (Berzowsky *et al.*, 1982). In addition, in most protocols hybridoma supernatants are screened and selected by measuring their reactivities with the intact macromolecule, and not with peptides. Peptide-reactive antibodies might then be missed in the final selection (Camera *et al.*, 1988).

The specificity of the binding of monoclonal antibodies to peptides seems to be relatively low. This has been assessed by replacing single amino acids within a peptide recognized by an anti-virus antibody (Geysen *et al.*, 1984), and thus synthesizing a broad set of peptides whose sequence was identical to that of the control fragment, except at one residue for which each of the other 19 other amino acids had been systematically inserted. Already for an hexapeptide, this method required the synthesis of more then 200 fragments, and many more for a longer fragment (Geysen *et al.*, 1986). Few attempts on the contrary have been made to investigate the effect of multiple substitutions within the epitope (Mateu *et al.*, 1992).

In a previous investigation, we defined the epitope recognized by an anti-P170 mouse monoclonal antibody using as antigens a panel of 12- to 18-mer synthetic peptides mimicking selected sequences for the protein. The 15-mer active fragment was then further investigated by testing the reactivity of the antibody on all possible overlapping 5- to 9-mer fragments of this peptide, as prepared by the multi-pin synthesis. (Cianfriglia *et al.*, 1995). In addition, a second monoclonal antibody reacting with the same peptide was isolated and partially investigated by the same approach (Cianfriglia, personal commun.).

The comparison of the reactivities of this latter monoclonal antibody with the set of pin-bound fragments suggests that three to four amino acid residues within the peptide might be critical for recognition, while the others are relatively unrelevant. We try now to assess whether a peptide of the same length comprising only the former few residues at the right position might mimic the epitope recognized by the antibody. Thus we replaced simultaneously from two to four amino acids in the peptide, and retested the antibody on this restricted number of fragments presenting multiple substitutions at predetermined positions, in order to confirm experimentally the validity of the theoretical approach, based on the analysis of the binding data on the peptide fragments.

Materials and Methods

The anti-P170 monoclonal antibody was kindly provided by Dr. Cianfriglia. It was obtained by using immunization protocols, somatic-cell fusion, and hybrid-selection procedures as previously de-

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scribed (Cianfriglia *et al.*, 1994). The specificity of this monoclonal antibody to human MDR cells was determined by testing the supernatant on a panel of cells expressing various relative levels of resistance. The antibody was reactive in ELISA with a 15-mer synthetic peptide selected from P170 sixth extracellular loop (Chersi, unpubl. results).

Peptides were synthesized on polypropylene rods (Geysen *et al.*, 1984), according to the instructions of the manufacturer (Chiron Mimotopes Pty Ltd, Australia). At the end of the synthesis, pins were treated for 2 hours with a mixture of trifluoroacetic acid : anisole 19:1 v/v in order to deprotect side-chain reactive groups, then washed with methanol, and dried in the air. The reactivity of pin-bound peptides with the monoclonal antibody was then assayed by a modified immunoenzymatic test: briefly, pins were immersed into wells of a microtiter plate filled with 150 µl of the anti-P170 monoclonal antibody , and allowed to react for 120 min. Pins were then washed several times in a tray with PBS containing 0.2% Tween 20, 0.5% bovine serum albumin (BSA) then reacted in the wells of a second plate with 150 µl of a 1:2000 dilution of goat anti-mouse IgG-peroxidase. After 60 min, pins were washed again and allowed to react in the wells of a third microtiter plate with 150 µl of a peroxidase substrate (*o*-phenylendiamine and hydrogen peroxide). The reaction was stopped after 5 minutes by addition of 50 µl of 4 N sulfuric acid, and the absorbancies of the solutions measured at 492 nm in a microtiter reader (Immunella GDV, Italy).

Results and Comments

We first examined the binding values of the monoclonal antibody to all overlapping penta- to nonapeptides, as derived from the ELISA assays on pins (Table I). This analysis provided a first information about the structure of the epitope. However, for this first comparison of binding data, ambiguities may arise from the fact that the variation in activity between two members of the same group, overlapping by all but one residue, could be ascribed either to the loss of an unrelevant residue at one terminus, or to the addition of a key residue at the other.

Table I. Binding affinities of the monoclonal antibody for fragments derived from peptide AYLVAHKLMSFE.

5-mer fragments												
A	Y	L	V	A								–
	Y	L	V	A	H							–
		L	V	A	H	K						240
			V	A	H	K	L					200
				A	H	K	L	M				130
					H	K	L	M	S			100
						K	L	M	S	F		260
							L	M	S	F	E	60
6-mer fragments												
A	Y	L	V	A	H							–
	Y	L	V	A	H	K						260
		L	V	A	H	K	L					490
			V	A	H	K	L	M				130
				A	H	K	L	M	S			100
					H	K	L	M	S	F		200
						K	L	M	S	F	E	60
7-mer fragments												
A	Y	L	V	A	H	K						230
	Y	L	V	A	H	K	L					240
		L	V	A	H	K	L	M				320
			V	A	H	K	L	M	S			180
				A	H	K	L	M	S	F		230
					H	K	L	M	S	F	E	60
8-mer fragments												
A	Y	L	V	A	H	K	L					260
	Y	L	V	A	H	K	L	M				310
		L	V	A	H	K	L	M	S			340
			V	A	H	K	L	M	S	F		280
				A	H	K	L	M	S	F	E	40
9-mer fragments												
A	Y	L	V	A	H	K	L	M				300
	Y	L	V	A	H	K	L	M	S			280
		L	V	A	H	K	L	M	S	F		610
			V	A	H	K	L	M	S	F	E	60

5-mer fragments

The antibody (Table I) displays already a moderate activity on some of the 5-mer fragments, i.e. LVAHK, VAHKL, KLMSF. Lysine 7 is the only residue which occurs in all reactive fragments.

6-mer fragments

The first active fragment is YLVAHK: the activity increases in the next term, LVAHKL, either because of loss of Tyr 2, or addition of Leu 8, or both.

Next two residues, Met and Ser, seem unrelevant, while Phe 11 slightly increases activity. Addition of Glu 12, more likely than loss of His 6, abolishes activity.

7-mer, 8-mer fragments

All fragments, except those that contain Glu, are moderately active. Indeed, the longer the chain, the higher the probability that essential residues are represented in the epitope.

9-mer fragments

The most active nonapeptide is LVAHKLMSF. Its high activity against the preceeding term might be due either to the loss of Tyr 2, or, more likely, to the presence of Phe 11. This 9-mer peptide (pos. 3–11) was then assumed as the reference for measuring the activities of the antibody on modified fragments.

Comparison of progressively elongated peptide chains provides additional information about essential residues. In this case, every change of binding affiinity can be ascribed to the effect of the added residue.

All peptides of the first group (Table II) are active, the best reactivity being exhibited by the hexapeptide LVAHKL. Addition of Tyr 2 decreases activity. Ala 1 seems unrelevant.

All peptides in the second group are active. Addition of Leu 3 to the octapeptide VAHKMSF sharply increases activity. Leu 3 might be then an important key residue for antibody recognition.

No one of the peptides in group 3, lacking Leu 3, is particularly active. In group 4, there is a sharp increase of activity adding Leu 9 to the pentapeptide LVAHK. Next two residues (Met, Ser) appear unrelevant. Phe 11 sharply increases again the binding affinity. The best peptide is by far LVAHKLMSF.

From all these data, we could presume that there are four residues that function as main residues for antibody recognition: Leu 3, Lys 7, Leu 8, and Phe 11. Any peptide containing two or three of those amino acids at the right place might then react with the antibody, almost independently from the nature of residues occupying non-relevant positions, while peptides modified at those amino acids might not be recognized.

To test this hypothesis, we synthesized a restricted number of peptides closely related to the nonapeptide displaying in ELISA the best binding activity, i.e. Leu Val Ala His Lys Leu Met Ser Phe (peptide A), which corresponded to residues 3–11 of peptide 49 (Table III). For this nonapeptide, and peptides derived from it, a new numbering of amino acids was introduced, being Leu 1, and Phe 9.

Peptides B and C derived from this first by substituting 2 of the predicted 4 key amino acid resi-

Table II. Binding affinities of the antibody for progressively elongated fragments derived from peptide AYLVAHKLMSFE.

1) At the amino terminus	
V A H K L	200
L V A H K L	490
Y L V A H K L	240
A Y L V A H K L	260
K L M S F	260
H K L M S F	200
A H K L M S F	230
V A H K L M S F	280
L V A H K L M S F	610
2) At the carboxy terminus	
V A H K L	200
V A H K L M	130
V A H K L M S	180
V A H K L M S F	280
V A H K L M S F E	60
L V A H K	240
L V A H K L	490
L V A H K L M	320
L V A H K L M S	340
L V A H K L M S F	610
L V A H K L M S F E	30

Table III. Sequence of modified peptides and corresponding binding values in ELISA.
A is the control nonapeptide exhibiting the best binding in ELISA.
X is an unrelevant peptide used as a negative control (background).
Unmodified amino acid residues are in **bold** characters.

										homology to A	% Binding (min-max)
A)	**L**	**V**	**A**	**H**	**K**	**L**	**M**	**S**	**F**		100%
B)	**L**	**V**	**A**	**H**	**K**	T	**M**	**S**	G	77%	67 to 73
C)	G	**V**	**A**	**H**	**K**	T	**M**	**S**	**F**	77%	72 to 82
D)	G	**V**	**A**	**H**	G	T	**M**	**S**	**F**	66%	4 to 6
E)	**L**	G	Q	A	**K**	**L**	T	**S**	**F**	55%	85 to 96
F)			Q	A	**K**	**L**	T	**S**	**F**	57%	62 to 67
X)	A	G	A	G	A	G	A	G	A	11%	

The test was performed in triplicates. The binding of the antibody on pin-bound peptide A, as determined by the ELISA test, (mean: 0.681) was corrected for the background (peptide X: 0.100) and assumed as 100%. The binding of the other pin-bound peptides was then referred to this value.

dues. Lys 5, however, was left unmodified in both fragments. In peptide D, also this key residue was replaced.

Peptide E (Leu Gly Gln Ala Lys Leu Thr Ser Phe) had four amino acid substitutions, as compared to the first fragment: Gly for Val, at pos. 2; Gln for Ala, at pos. 3; Ala for His, at pos. 4; Thr for Met, at pos. 7. All those positions were considered as relatively unrelevant for antibody binding. Leu 1, Lys 5, Leu 6, and Phe 9, considered as key positions, remained thus unmodified.

Peptide F derived from E, with two deletions at pos. 1 and 2 (Leu and Gly).

The last peptide (AGAGAGAGAG) was the negative control.

The binding data on pin-bound peptides, as reported in Table III, indicate that, assuming as 100% the activity of the antibody on control peptide A, the bindings of peptide B and C, modified at two of the 4 predicted key residues (homology to A: 77%), were remarkably lower, while in peptide D, containing an additional replacement at a third key residue (Lys) , the activity dropped almost to zero (4–6%). In peptide E, where as much as 4 residues were substituted, (homology to A: 55%), the activity was however still in the order of 90% . Also the 7-mer peptide F, although lacking Leu 1 (but possessing Lys 5, Leu 6, and Phe 9), was still moderately active (65% of the control).

Thus, the epitope recognized by the monoclonal antibody might be formulated as

Leu-X-X-X-Lys-Leu-X-X-Phe

being Leu 1, Lys 5, Leu 6, and Phe 9 almost essential for recognition, i.e. amino acids whose replacement by other residues results in a remarkable loss of antibody binding activity (Geysen *et al.,* 1985). Thus, the analysis of the binding data on all 5- to 9-mer fragments of the peptide, and the comparison of the reactivities of all individual fragments, allow to predict which residue can be replaced without altering the antigenic properties of the epitope, and which plays a critical role in the recognition. A very limited number of binding assays on fragments presenting simultaneous multiple substitutions at predetermined positions is then sufficient to confirm the prediction.

The fact that as much as 4 out of 9 residues within the peptide could be simultaneously replaced without significant loss of activity confirms the scarse specificity of antibody binding to peptides. This statement seems in contrast with their high selectivity for complex antigens as proteins (Alexander *et al.,* 1983; Rowlands *et al.,* 1983). It is known however that the large majority of anti-protein monoclonal antibodies are directed against conformational antigenic sites, and antibody specificity is dictated by both the sequence and the conformation at the region of binding. A peptide is thought as to represent only a part of this determinant, with the involvment of just few amino acids in the process, and the absence of the original three-dimensional structure of this incomplete epitope further compromises a selective, proper interaction. It should remainded, on the other side, that antibodies produced against peptides often crossreact with proteins displaying relatively scarse homology, (Chersi *et al.,* 1989), making thus unreliable their use for the specific recognition of closely related proteins. Accordingly, the use of peptides for testing the specificity of monoclonal antibodies seems to be subjected to similar restrictions and ambiguities.

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Alexander H., Johnson D., Rosen J., Jerabek L., Green N., Weissman I. L., and Lerner R. A (1983), Mimicking the alloantigenicity of proteins with chemically synthesized peptides differing in single amino acids. Nature **306,** 697–699.

Berzofsky J. A., Buckenmeyer G. K., Hicks G., Gurd F. R. N., Feldman R. J., and Minna J. (1982), Topographic antigenic determinants recognized by monoclonal antibodies to sperm whale myoglobin. J. Biol. Chem. **257,** 3189–3198.

Camera M., Muratti E., Trinca M. L., and Chersi A. (1988), Do monoclonal antibodies recognize linear sequential determinants? Z. Naturforsch. **43c,** 967–970.

Chersi A., Romano T. F., and Chillemi F. (1989), Preparation and isolation of antibodies to human MHC class II alpha chains by aid of synthetic peptides. Z. Naturforsch. **44c,** 813–818.

Cianfriglia M., Willingham M. K., Scagliotti G. V., Frasca G., and Chersi A. (1994), Identification of a linear human-specific epitope in the fourth loop of theP-glycoprotein extracellular domain by MM4.17 murine monoclonal antibody to human multi-drug-resistant cells. Int. J. Cancer **56,** 153–160.

Cianfriglia M., Romagnoli G., Tombesi M., Poloni F., Falasca G., Di Modugno F., Castagna M., and Chersi A. (1995), P-Glycoprotein epitope mapping: the murine monoclonal antibody MM6.15 to human multidrug-resistant cells binds with three distinct loops in the MDR1-P.-glycoprotein extracellular loop. Int. J. Cancer **61,** 142–147.

Geysen H. M., Meloen R. H, and Barteling S. J. (1984), Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81,** 3998–4002.

Geysen H. M., Barteling S. J., and Maloen R. H. (1985), Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proc. Natl. Acad. Sci. USA **82,** 178–182.

Geysen H. M., Rodda S. J., and Mason T. J. (1986), A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol. Immun. **23,** 709–715.

Mateu M. G., Andreu D., Carreno C., Roig X., Cairo J. J., Camarero J. A., Giralt E., and Domingo E. (1992), Non-additive effects of multiple amino acid substitutions on antigen-antibody recognition. Eur. J. Immunol. **22,** 1385–1389.

Rowlands D. J., Clarke B. E., Carroll A. R., Brown F., Nicholson B. H., Bittle J. L., Houghten R. A., and Lerner R. A. (1983), Chemical basis of antigenic variation in foot-and-mouth disease virus. Nature **306,** 694–697.