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Epitope diversity of angiotensin II analysed with monoclonal antibodies

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SUMMARY

The antigenic heterogeneity of angiotensin II (AII) was studied with monoclonal antibodies. Twelve antibodies were produced and characterized. Association constants for AII varied from $1{\cdot}2 \times 10^8$ to $1{\cdot}1 \times 10^{10}$ M^{-1}. The fine specificity of the Mab was studied by immunoenzymoassay using solid-phase AII. Using AII analogues in binding inhibition experiments, three groups of specificity could be characterized: (1) five antibodies reacted only with peptides in which phenylalanine is the carboxy terminal aminoacid; for two of these antibodies, tyrosine^4 is closely associated with the binding site, since iodine labelling suppresses reactivity; (2) two antibodies also required phenylalanine in position 8, but, in addition, reacted with AI, a decapeptide in which phenylalanine is not terminal; (3) five antibodies reacted with analogues in which phenylalanine had been substituted for another amino acid. In addition, studies in which binding of a biotinylated Mab to solid-phase AII was analysed in the presence of various unlabelled Mab suggest further antigenic heterogeneity of AII.

INTRODUCTION

The octapeptide angiotensin II (AII) is the major effector of the renin–angiotensin system. It induces vasoconstriction and aldosterone secretion via receptors located, respectively, on blood vessels (Regoli, Park & Rioux, 1974) and adrenal gland (Capponi & Catt, 1979). Thus, it plays a key role in blood pressure regulation and sodium balance, but specific receptors have also been detected in other organs (Devynck & Meyer, 1976; Sraer *et al.*, 1974; Campanile *et al.*, 1982). AII is produced through cleavage of a carboxy (C)-terminal dipeptide from its inactive precursor, angiotensin I (AI), by angiotensin-converting enzyme (ACE). It is converted by aminopeptidases to angiotensin III (desAsp^1-AII) which also stimulates aldosterone secretion but has lost most of its vasoactive properties, and further broken down to shorter inactive peptides. Using peptide analogues of AII, it has been established that the C-terminal amino acid phenylalanine (Phe^8) is crucial for biological activity (Bumpus *et al.*, 1961; Smeby *et al.*, 1962), although such carboxy-terminus substituted peptides can still bind to AII receptors. However, more detailed information concerning binding of AII to the receptor complex is so far unavailable.

The immunological approach to the final effectors of the renin–angiotensin system is very attractive, but has been hampered by the lack of specificity of polyclonal reagents. Indeed, most conventional antisera raised against AII are reactive mainly with the C-terminal moiety of AII and AIII (Valloton, 1970; Nussberger *et al.*, 1983; 1984), and show variable but definite cross-reactivity with AI (Haber *et al.*, 1969). In order to study the repertoire and physiological significance of epitopes on the AII molecule, we have raised 12 high-affinity monoclonal antibodies (Mab) against AII. We report here on their characterization and fine specificity. They define at least four different epitopes, which may be of interest to set up specific immunoassays and to analyse more precisely the molecular structures involved in receptor binding.

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MATERIALS AND METHODS

Monoclonal antibody production

Ten BALB/c mice were immunized subcutaneously three times at 3-week intervals with 250 μg of Val^5-AII (Hypertensin CIBA, Basel, Switzerland) coupled using carbodiimide to keyhole limpet haemocyanin (KLH) in a w/w ratio of 40:1, and emulsified in Freund's complete adjuvant. Responders were hyperimmunized on 3 consecutive days by intraperitoneal (i.p.) injections of 200 μg of AII-KLH in saline. The spleen was removed on the fourth day. Fusion of splenocytes (Kohler & Milstein 1975) with NS1 myeloma cells and selection of hybrids in HAT medium were performed as previously described (Ronco *et al.*, 1984). The supernatants of hybrid-containing wells were tested by immunoenzymoassay (IEA) and liquid-phase radioimmunoassay (RIA). Cells producing antibodies of desired specificity were cloned by limiting dilution. Immunoglobulin class and subclass were determined by Ouchterlony analysis of supernatants using commercial antibodies purchased from Bionetics (Kensington, MD). Isoelectric points (pI) were measured as previously described (Ronco *et al.*, 1984).

When substantial amounts of antibody were needed, the cloned cells were inoculated i.p. in BALB/c mice pretreated with Pristane.

Detection of anti-AII antibodies

IEA was performed on polycarbonate microtitre plates (Flow Laboratories, Puteaux, France) coated with AII coupled to ovalbumin (OVA) in a ratio of 400:1 using carbodiimide. Plastic wells were coated by incubation for 4 hr at 37° with AII-OVA (1 μg/ml AII) in 0·1 M carbonate–bicarbonate buffer, pH 9·4. Control wells were prepared using carbodiimide-treated OVA adjusted in the same buffer to the concentration of the carrier in AII-OVA solution (approximately 0·035 μg/ml). After washing in phosphate-buffered saline (PBS) containing 0·1% Tween, wells were incubated with the supernatants followed by peroxidase-conjugated sheep anti-mouse IgG.

RIA was carried out using Ile^5-AII radiolabelled with ^{125}I to a specific activity of 2200 Ci/mmole (NEN, Dreiech, West Germany). Briefly, 25 μl supernatant were incubated with 25 μl PBS containing approximately 20,000 c.p.m. ^{125}I-AII for 3 hr at room temperature. Separation of bound from free hormone was obtained by incubating the mixture overnight at 4° with 25 μl sheep anti-mouse IgG, followed by 25 μl polyethylene glycol 6000 (PEG 6000, MSD, Munich, West Germany) at a final concentration of 5% (w/v). The precipitates were washed in 5% PEG and counted in a Packard gamma counter.

Monoclonal antibody affinity

Determination of Mab affinity constants (K_A) for human ^{125}I-AII (Ile^5-AII) was performed by analysis of the binding curves according to Scatchard (1949). Briefly, constant amounts of Mab were incubated in the presence of varying concentrations of ^{125}I-AII for 30 min at 4°. The reaction was performed in 0·15 ml of PBS containing 0·1% BSA. Bound AII was immunoprecipitated using sheep anti-mouse Ig in the presence of PEG at a final concentration of 10%. Precipitates were then retained on 0·45 μm GV-type filters (Millipore, Saint Quentin, France) and washed with 1 ml PBS containing 10% PEG. Blanks set up by incubating a non-specific mouse IgG with varying concentrations of ^{125}I-AII amounted to 5 ± 2% of the specific binding.

The consequences of AII-labelling on Mab affinity constants were evaluated by competition studies. Using unlabelled AII as a competitor for ^{125}I-AII, it was possible to determine the association constants (Kc) for the competitor using a modified version of the formula of Cheng & Prussoff (1973):

$$Kc = \frac{1 + K_A \times (^{125}\text{I-AII})}{IC_{50}}$$

IC_{50}, the concentration necessary to inhibit 50% of the binding, is determined from the plot of the isotopic intensity (c.p.m.) versus the logarithm of the competitor concentration; K_A is the association constant of Mab studied for ^{125}I-AII; (^{125}I-AII) is the labelled AII concentration adjusted at the value corresponding approximately to dissociation constants (K_D).

The association constants of selected Mab were measured using (^{3}H-Tyr^4)-Ile^5-AII obtained from Dr Vanhove (Service des Molècules Marquées, CEA, Saclay, France). It was purified by high-pressure liquid chromatography to a specific radioactivity of 57 Ci/mmol. Its chemical purity was controlled by a rapid spectral detector (LKB, Orsay, France).

Table 1. Angiotensin II (AII) analogues

AII	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
(Val^5)-AII	Asp–Arg–Val–Tyr–*Val*–His–Pro–Phe
AI	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–*His–Leu*
AIII	— Arg–Val–Tyr–Ile–His–Pro–Phe
(DesAsp^1-Ile^8)-AII*	— Arg–Val–Tyr–Ile–His–Pro–*Ile*
Sar^1–Ile^8-AII	*Sar*–Arg–Val–Tyr–Ile–His–Pro–*Ile*
Sar^1–Ala^8-AII	*Sar*–Arg–Val–Tyr–Ile–His–Pro–*Ala*

AI, angiotensin I; AII, angiotensin II; AIII, angiotensin III.
* AIII inhibitor.

Monoclonal antibody specificity

The fine specificity of the Mab was analysed in inhibition studies using the IEA.

In a first set of experiments, antibody binding was tested in the presence of various peptide inhibitors listed in Table 1. In preliminary studies, for each antibody tested, limiting amounts of antibody and solid-phase adsorbed antigens were determined by reacting serial two-fold dilutions of supernatant with plastic wells coated with 15–1000 ng/ml AII. Inhibition experiments were then performed by preincubating the supernatant with increasing concentrations of each peptide from 1×10^{-11} to 1×10^{-5} M (final concentration) for 1 hr at 20°. Two-hundred μl of the mixture were allowed to react with AII-coated wells, and the amount of solid-phase bound Mab was estimated by adding peroxidase-conjugated sheep anti-mouse IgG as described above. For each inhibitor, the IC_{50} ratio was calculated as the concentration of inhibitor leading to 50% inhibition of AII binding divided by the concentration of Ile^5-AII giving 50% inhibition.

In a second set of experiments, solid-phase AII binding of Mab labelled with Biotin according to Subbarao *et al.* (1983) was assessed in the presence of increasing amounts of various unlabelled Mab. Optimal concentrations of solid-phase AII and biotinylated Mab were estimated as described above. Cross-inhibition experiments were then performed by preincubating for 1 hr at 20° the biotinylated Mab with the same or each of the other Mab in ascites serially diluted 1/4 from 1/10 to 1/10,240. The amount of solid-phase bound biotinylated Mab was determined by further incubation with peroxidase-labelled streptavidin (Amersham, Les Ulis, France), followed by orthophenylenediamine.

RESULTS

Monoclonal antibody production

459/480 seeded wells contained hybrids after 10–20 days of culture. Twenty-two produced Mab reactive in ELISA with AII-OVA but not OVA alone. In 10 cases binding to AII-OVA was not inhibited by an excess of AII added in the supernatant, thus indicating that those Mab were directed to neodeterminants induced by coupling of AII to OVA. Ten of the 12 remaining Mab bound ^{125}I-AII in RIA. Only the hybrids producing those 12 Mab were cloned, kept viably frozen and expanded in ascites. All Mab were of IgG1 subclass. The isoelectric points ranged from 5·7 to 6·8.

Table 2. Measurements of affinity for AII of 10 monoclonal antibodies

	K_A M^{-1}*	r†	Kc M^{-1}‡	Delta error (%)§
110	$1{\cdot}3 \pm 1{\cdot}5\ 10^9$	0·991	$4{\cdot}5\ 10^8$	4·06
211	$7{\cdot}6 \pm 0{\cdot}3\ 10^8$	0·882	$7{\cdot}0\ 10^8$	7·3
91	$1{\cdot}9 \pm 0{\cdot}9\ 10^9$	0·824	$3{\cdot}8\ 10^9$	7
11¶	$1{\cdot}6 \pm 0{\cdot}5\ 10^8$	0·945	ND**	
394¶	$1{\cdot}2 \pm 0{\cdot}2\ 10^8$	0·979	ND	
199	$1{\cdot}2 \pm 0{\cdot}2\ 10^9$	0·974	$2{\cdot}0\ 10^9$	8
301	$8{\cdot}6 \pm 0{\cdot}8\ 10^8$	0·624	$2{\cdot}5\ 10^9$	3·2
76	$4{\cdot}2 \pm 0{\cdot}6\ 10^8$	0·903	$6{\cdot}1\ 10^8$	4
120	$2{\cdot}6 \pm 0{\cdot}2\ 10^8$	0·997	$3{\cdot}7\ 10^8$	9·7
392	$4{\cdot}8 \pm 0{\cdot}1\ 10^8$	0·935	$3{\cdot}4\ 10^9$	2·9
393	$1{\cdot}0 \pm 0{\cdot}8\ 10^9$	0·995	$1{\cdot}6\ 10^9$	3·8
133	$3{\cdot}4 \pm 0{\cdot}7\ 10^9$	0·956	$1{\cdot}1\ 10^{10}$	6·6

* K_A, association constant obtained from Scatchard plots.

† r, correlation coefficient of Scatchard plots.

‡ Kc, association constant of unlabelled AII (see text).

§ Delta error, standard error, expressed as percentage between theoretical and experimental curves.

¶ Monoclonal antibodies 394 and 11 do not bind ^{125}I-AII. Measurements were made using ^{3}H-AII.

** ND, not determined.

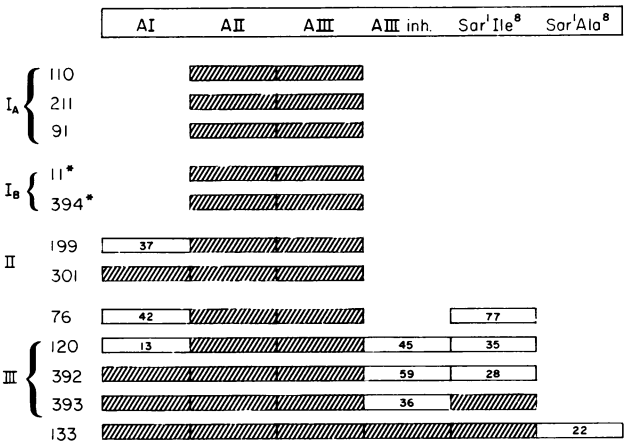

Figure 1. Summary of the specificities of 12 monoclonal anti-AII antibodies analysed with AII analogues. The peptide inhibitors used are listed horizontally at top of the figure. The 12 monoclonal antibodies are listed vertically on the left-hand side of the figure. Reactivity of a peptide with a Mab is characterized by its IC_{50} (i.e. ratio of analogue/AII concentrations giving 50% inhibition of binding). (■) $1 < IC_{50} < 10$; (□) $10 < IC_{50} < 100$. (*), not reactive with ^{125}I-AII.

Monoclonal antibody affinity

It was found that ^{125}I-AII bound to the different Mab tested in a saturable way. Scatchard analysis (Scatchard, 1949) of the saturation curves demonstrated a homogeneous class of binding sites. K_A values ranged from $2{\cdot}6 \times 10^8$ to $3{\cdot}4 \times 10^9$ M^{-1}. From the experimental IC_{50} determination, the Kc values (i.e. association constants for native AII) were in good agreement with those obtained from the Scatchard plots, thus demonstrating that iodination of AII did not significantly change its reactivity with the Mab. Mab 11 and 394 were unable to bind ^{125}I-AII. However, effective binding was obtained using ^{3}H-AII. Results are summarized in Table 2. In addition, for comparison purposes, we tested ^{3}H-AII with Mab 133 and found the same K_A (data not shown) as that obtained from competition studies.

Monoclonal antibody specificity

The immunological cross-reactivities of the Mab for structural analogues of Ile^5-AII were determined by ELISA inhibition studies. The analogues were selected either for their physiological role (Val^5-AII, AIII, AI) or because of a substitution of one or more residue(s) at positions known to be crucial for biological activity. Reactivity for Val^5-AII and Ile^5-AII was identical for all Mab. Results of inhibition experiments were expressed for each analogue as IC_{50} ratios. As shown in Fig. 1, those studies allowed us to classify the 12 Mab into three groups. Representative inhibition experiments for one Mab from each group are illustrated in Fig. 2.

The first group comprises five Mab which recognize only AII and AIII. Their binding site specifically involves the C-terminus amino acid phenylalanine (Phe), since they are not reactive with peptides substituted at this position. Furthermore, binding to AII is not inhibited by AI, thus indicating that the reactive epitope is dramatically altered when two amino acids are added at the C-terminal extremity. Although inhibition profiles are very similar for all Mab of this group, there are at least two subgroups of binding sites since Mab 11 and Mab 394 are not reactive with ^{125}I-AII, but reactive with ^{3}H-AII.

The two Mab of the second group are also specific for the C-terminus Phe, but are likewise reactive with AI, albeit to a lesser degree.

The third group is made up of five Mab which share a broad spectrum of reactivity. The prominent feature is reactivity with carboxy-terminus substituted peptides, such as (desAsp^1 Ile^8)-AII, (Sar^1 Ile^8)-AII and (Sar^1 Ala^8)-AII. The inhibition profiles suggest that the binding sites involve amino acids more internal than Phe. However, as estimated by IC_{50} ratios, their specificity still involves the C-terminal moiety of AII, since Mab of this group have greater affinity for AIII than for AIII inhibitor. The epitope defined by Mab 133 is difficult to ascribe to either C- or N-terminal moiety because all analogues tested induce similar inhibitions.

In a second approach to analyse the fine specificity of Mab anti-AII, binding of biotinylated Mab was studied in the presence of serial dilutions of the same or each of the other Mab. Unfortunately, only Mab 110 and Mab 199 could be satisfactorily labelled with biotin. Binding of biotin-labelled Mab 110 to solid-phase AII (Fig. 3a) was completely inhibited by unlabelled Mab 110, 211 and 199. Mab 11, 91, 394 and 301, which also require a phenylalanine in position 8, could not produce more than 50% inhibition of binding. Mab from Group 3 were poor inhibitors, except for Mab 133 which could compete efficiently with Mab 110. Similar results were obtained using biotin-labelled Mab 199 (Fig. 3b).

DISCUSSION

Although considerable efforts have been devoted to the production of polyclonal antisera specific for AII, most of them cross-react with AIII and to some extent with the precursor decapeptide AI. The hybridoma technology used in this work confirmed

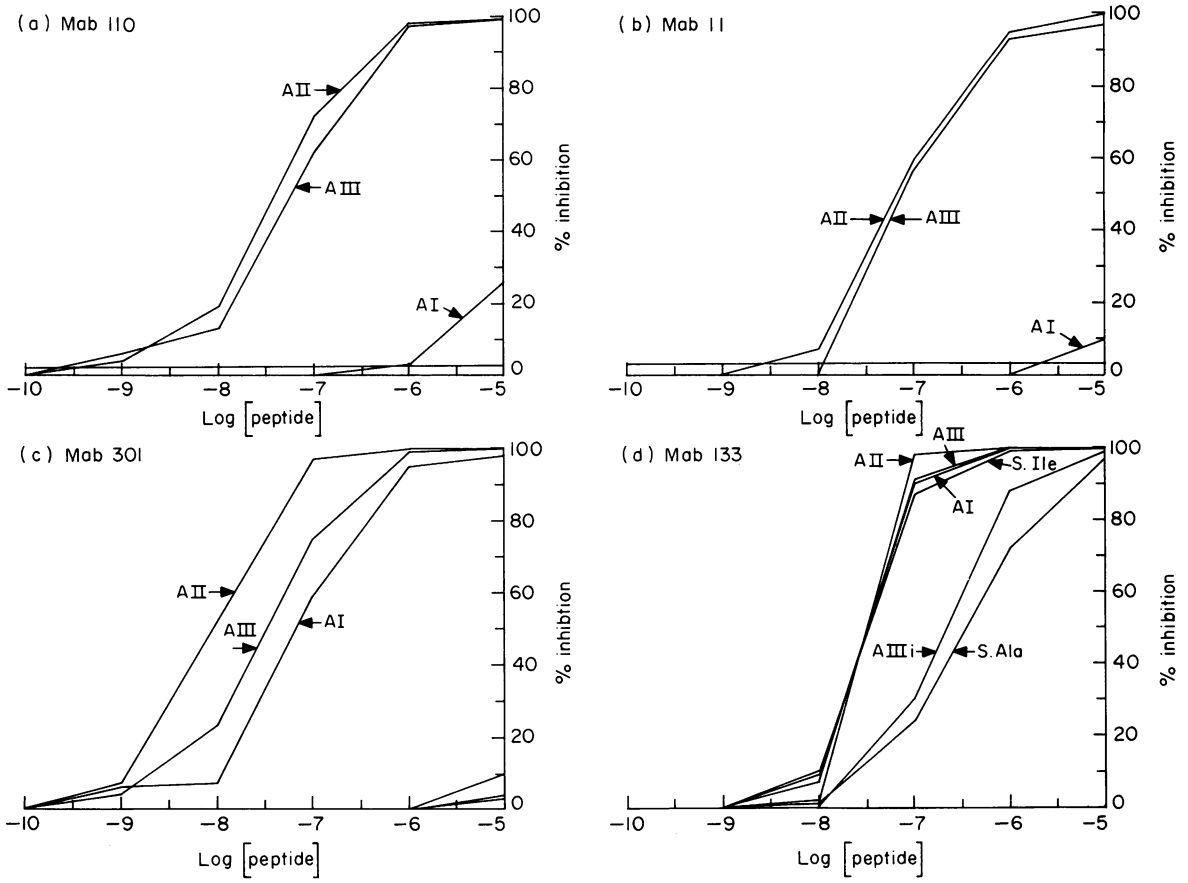

Figure 2. Competitive inhibition of binding of (a) Mab 110, (b) Mab 11, (c) Mab 301 and (d) Mab 133 to solid-phase AII by increasing amounts of AII analogues. Percentage inhibition is shown on the ordinate as a function of the concentration of AII analogue. Each individual point is the average of triplicates. (a) and (b) Horizontal line indicates lack of inhibition by the following peptides: AIII inhibitor, Sar^{1}Ala^8-AII, Sar^{1}Ile^8-AII. (c) Less than 10% inhibition was observed with AIII inhibitor, Sar^{1}Ala^8-AII and Sar^{1}Ile^8-AII.

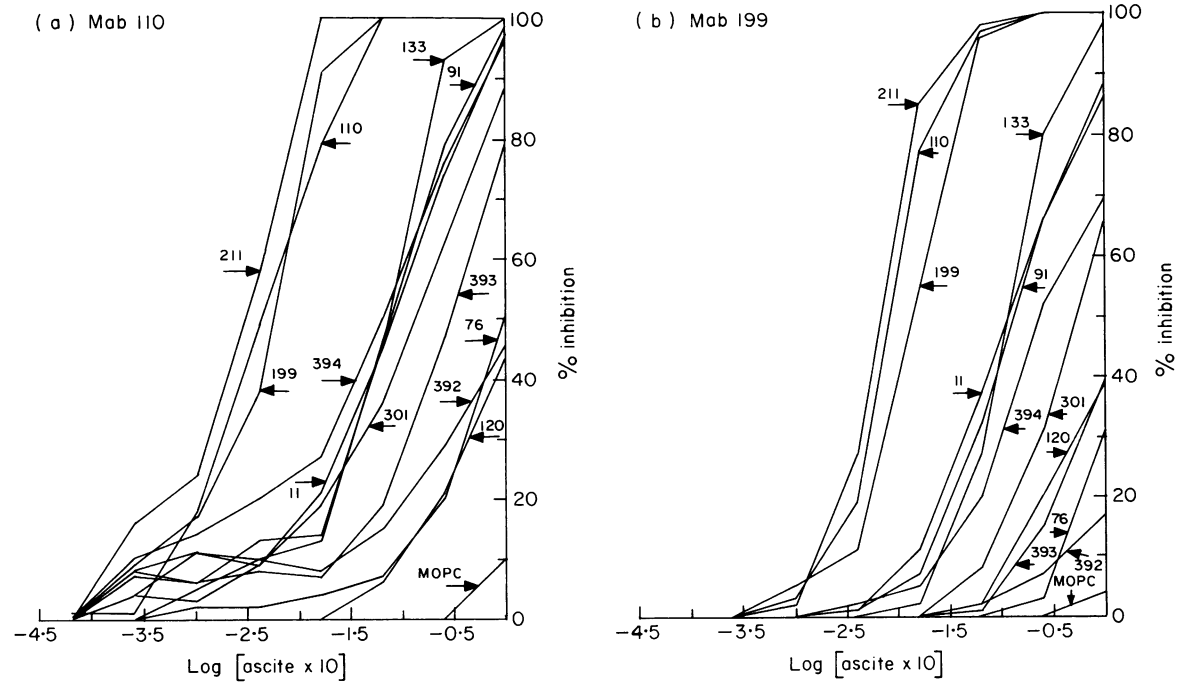

Figure 3. Competitive inhibition of binding of biotinylated (a) Mab 110 and (b) Mab 199 to solid phase AII by increasing concentrations of unlabelled monoclonal anti-AII antibodies. Percentage inhibition is shown on the ordinate as a function of unlabelled Mab concentration. MOPC, control Ig2a MOPC 173 devoid of known antibody specificity.

the heterogeneity of the anti-AII immune response, and led to the production and characterization of 12 Mab anti-AII reactive with a variety of epitopes and exhibiting high binding affinity.

The epitope diversity of the AII molecule is first demonstrated by inhibition studies performed with peptide analogues of AII. By comparison of inhibition profiles, it was possible to classify the 12 Mab into three groups which showed quite distinct specificities. Monoclonal antibodies from Groups 1 and 2 were only reactive with peptides in which the C-terminal amino acid is phenylalanine, since substitution for another amino acid led to unreactive analogues. Mab from Group 1 required Phe 8 as the terminal amino acid, whereas Mab from Group 2 were still reactive with AI, which carries two additional amino acids in positions 9 and 10. Mab from Group 3 were defined by variable reactivity with peptides substituted in position 8, but, except for Mab 133, the patterns of inhibition suggest that determinants identified by these Mab are probably located on the carboxy-terminal moiety of AII.

However, the repertoire of epitopes defined by the Mab that we have produced seems to be broader than suggested by peptide inhibition studies. This is first illustrated by radioimmunoassays using labelled AII which show that Mab 11 and Mab 394 do not react as the other Mab of the same group with ^{125}I-AII, but only with ^{3}H-AII, although they exhibit similar reactivity with AII analogues. This behaviour demonstrates that the epitope involved is dramatically modified by ^{125}I-labelling of tryrosine residue at position 4, although it also includes the C-terminus phenylalanine. This epitope probably encompasses both amino acids, in accordance with the spiral model described for AII by Smeby *et al.* (1962) in which there is close apposition of the two aromatic rings of Tyr^{4} and Phe^{8}. In second place, inhibition profiles of biotinylated Mab 110 and 199 by other unlabelled Mab were in agreement with the results of the peptides' inhibition analysis, but also suggested greater epitope diversity than anticipated. For instance, Mab 199 inhibited binding of biotinylated Mab 110, whereas Mab 110 and 211 inhibited binding of biotinylated Mab 199. Also, Mab 133, which has the 'broadest specificity' when studied with AII analogues, inhibited binding of biotinylated Mab 110 and Mab 199. In addition, Mab 11 and 394, which share similar analogues inhibition patterns but can be differentiated by their inability to bind ^{125}I-AII, are poorer inhibitors than Mab 211 against biotinylated Mab 110. These results should, however, be interpreted with caution since steric hindrance and binding affinity can intervene. The role of steric hindrance appears unlikely, since symetrical inhibition profiles were obtained with biotinylated Mab 110 and 199. On the other hand, affinity constants that vary over two orders of magnitude may contribute significantly to the patterns obtained. These results are in accordance with findings by Schroer *et al.* (1983) who demonstrated that each of 18 Mab raised against insulin recognized a unique site.

The variety of Mab-defined epitopes on the AII molecule provides an attractive tool to analyse the molecular structures involved in receptor binding. Using such an approach, Moyle, Ehrlich & Canfield (1982) could localize the receptor-binding site on human chorionic gonadotropin with two Mab which inhibited the formation of the hormone–receptor complex and blocked induction of a biological response. Such Mab can also be utilized to produce anti-idiotypic antibodies with cross-reactivity towards the hormone receptor (Sege & Peterson, 1978; Schreiber *et al.*, 1980; Wassermann *et al.*, 1982; Marasco *et al.*, 1982; Meo *et al.*, 1983). Experiments are in progress to determine whether some of our Mab could inhibit receptor binding of AII.

Finally, Mab from Group 1 which are Phe^{8}-specific and are not cross-reactive with AI, might be valuable for AII concentration measurements in patients treated with ACE inhibitors, a situation where AI levels are markedly increased.

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