Production and Characterization of Monoclonal Antibodies to Rat Angiotensinogen

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SUMMARY Three stable monoclonal antibodies to rat angiotensinogen were obtained by fusing myeloma cells with spleen cells from Balb/c mice injected with pure rat angiotensinogen. They were screened by their binding to pure iodinated angiotensinogen and to insolubilized angiotensinogen in a solid phase assay. The tilers of the three antibodies varied from 1/3500 to 1/35000, their dissociation constants from 2.5 x 10"[8]Mto3.8 x 10"[10] M, and the sensitivity of the assay ranged from 200 to 10 pmol of pure angiotensinogen. These monoclonal antibodies did not recognize either angiotensin peptides or angiotensinogen from other species, except for mouse angiotensinogen, which cross-reacted with the different antibodies from 0 to 25%. Rat cerebrospinal fluid angiotensinogen, plasma des-angiotensin I-angiotensinogen, and plasma angiotensinogen were equally recognized by these monoclonal antibodies. Contrary to what was observed for a polyclonal antiserum, the monoclonal antibodies failed to inhibit the renin-angiotensinogen reaction in vitro.
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KEY WORDS • angiotensinogen • monoclonal antibody • cerebrospinal fluid • des-angiotensin I • angiotensinogen • radioimmunoassay

SPECIFIC polyclonal antisera to rat angiotensinogen were recently obtained by conventional techniques and have been used to develop a radioimmunoassay (RIA)[1] for studying the role of angiotensinogen in controlling blood pressure,[2] as well as the hormonal regulation of angiotensinogen metabolism.[3]-"This source of antibodies is nevertheless limited in quantity, reproducibility, and homogeneity. For these reasons, we developed monoclonal antibodies to rat angiotensinogen. These antibodies were obtained by fusing mouse spleen cells immunized with pure angiotensinogen with mouse myeloma cells. The hybrid cells were cloned, and clones producing monoclonal antibodies to angiotensinogen were detected by RIA and solid phase assay. Mass production of antibodies was obtained by intraperitoneal injection of the hybrid cells into mice. Three monoclonal antibodies were compared with a polyclonal antiserum for their titer, affinity, specificity, and ability to inhibit the renin-angiotensinogen reaction. We also compared the immunoreactivity of each monoclonal antibody in relation to pure angiotensinogen, plasma angiotensinogen, plasma des-angiotensin I-angiotensinogen, and cerebrospinal fluid (CSF) angiotensinogen. All of these experiments provided new information about the rat angiotensinogen molecule and suggested that these monoclonal antibodies would be useful for immunohistochemical studies and immunopurification.

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Materials and Methods

Immunization

Adult Balb/c mice were injected subcutaneously with 7 /ig of angiotensinogen purified as previously described.[3] The specific activity equaled that calculated for pure angiotensinogen (23 £ig/mg protein). Angiotensinogen was emulsified in complete (first injection) or incomplete Freund's adjuvant (subsequent injections); mice were injected subcutaneously three times each week for 9 weeks, and a final injection was given intraperitoneally 3 days before the animals were killed.

Cell Fusion

We fused 10^8 spleen cells from an immunized mouse with 10^s cells of either the FO or AG 8-653 myeloma

cell lines by a method adapted from Fazekas et al.[6] The fusion was performed in the presence of 1.5 ml of 47.5% polyethylene glycol (MW 4000) for 90 seconds.

The cells were suspended in hypoxanthine aminopterin thymidine medium (HAT) and distributed into microculture plates. After 8 days of growth in HAT medium, supernatants from wells exhibiting cell growth were tested for the production of angiotensinogen-specific antibody.

Screening of Angiotensinogen Antibodies

The hybrid cells growing in HAT medium were tested for their angiotensinogen monoclonal antibody production by two different methods: 1) binding to pure ^{125}I-angiotensinogen, and 2) solid phase assay. The angiotensinogen antibodies were detected in the culture supernatants by their ability to bind pure iodinated rat angiotensinogen, by use of a procedure similar to that described for the direct RIA of rat angiotensinogen.' Bound and free angiotensinogen were separated by precipitation with polyethylene glycol (PEG, MW 8000, Fluka AG, Chemische-Fabrik, Fuchs, Switzerland).

For the solid phase assay, 2 /JL\ of a 2 pig/ml solution of pure angiotensinogen was adsorbed to the wells of a poly vinyl microtiter plate overnight at 4° C. After washing the microtiter plate, the supernatants of the hybridoma cultures were incubated for 2 hours at 37° C in the angiotensinogen-coated wells. The wells were again washed, and iodinated goat-antimouse IgG immunoglobulin was added and incubated for 2 hours at room temperature. After repeated washings, the wells were cut out and counted in a gamma counter.

Hybridoma Cloning and Expansion

Cultures producing antibodies to rat angiotensinogen were cloned and subcloned. Positive clones were injected intraperitoneally into Balb/c mice (5×10^7 cells per mouse). After a few days, serum and ascites fluid were collected. Mass production of one antibody (136) could not be obtained. Immunoglobulins from ascites or culture supernatants were purified by a two-step procedure with ammonium sulfate precipitation and DEAE cellulose chromatography, as previously described.[7]

Characterization of Monoclonal Antibodies

The isotype of each monoclonal antibody was determined with a protein A Sepharose column as described earlier.[8] Pure angiotensinogen was labeled by ^{12J}I with chloramine T according to Greenwood et al.[9] The RIA technique used for the characterization procedures (described below) has already been reported.' Briefly, 200 /i.1 of ^{123}I-angiotensinogen (10,000 cpm) in 0.1 M phosphate buffer, pH 7.5, containing 5 mM EDTA, was added at the same time to 200 /xl of monoclonal antibody in the same buffer with or without 100 /nl unlabeled angiotensinogen. After 24-hour incubation at 4° C, bound and free angiotensinogens were separated by 20% PEG in the presence of 1 mg bovine gammaglobulin. After centrifugation, the precipitate was counted in a gammacounter.

Serial dilutions of the ascites or hybridoma culture media were performed, and the dilution binding 50% of ^{125}I-angiotensinogen was calculated and used in subsequent procedures. The minimum detectable amount of angiotensinogen was established with each antibody by adding increasing amounts of pure angiotensinogen to the labeled angiotensinogen antibody mixture and measuring bound radioactivity displacement. The resulting standard curve built with pure angiotensinogen was compared to the curves obtained with dilutions of rat plasma angiotensinogen, rat cerebrospinal fluid (CSF) angiotensinogen, and des-angiotensin I-angiotensinogen. The last product was obtained after hydrolysis of rat plasma angiotensinogen by mouse submaxillary gland renin at 37° C for 2 hours in 0.2 M phosphate buffer, pH 6.5, and after dialysis before use, as described previously.'

The specificity for each antibody was tested by determining the ability of plasma angiotensinogen from different species, angiotensin I, II, and tetradecapeptide to displace the antibody ^{123}I-angiotensinogen binding.

Furthermore, the respective antibody association constants (K_a) were determined by using concentrations of pure angiotensinogen ranging from $10'^{"}$ M to $2 \times 10"^{7}$ M. The K_a were calculated according to Scatchard.[10]

Inhibition of the Renin-Angiotensinogen Reaction

The ability of monoclonal antibodies to inhibit the renin-angiotensinogen reaction was tested on a pool of binephrectomized rat plasma. Purified immunoglobulins were used for these experiments; 10 pd of plasma diluted 1/50 was preincubated for 24 hours at 4° C with decreasing dilutions of either polyclonal or monoclonal immunoglobulins in a final volume of 100 pd in 0.1 M phosphate buffer, pH 7.5, containing 1 mg/ml albumin. An excess of pure mouse submaxillary gland renin was then added in 0.2 M phosphate buffer pH 6.5, 35 mM EDTA, 1 mg/ml albumin, 3 mM phenylmethansulfonylfluorid (PMSF), in a final volume of 500 *fi*\. The mixture was incubated for 2 hours at 37° C, and the angiotensin I production was measured by RIA." Negative controls were performed by replacing the culture medium with an identical volume of buffer, and positive controls by adding purified polyclonal antibody.

Results

The two different fusions performed enabled 120 hybrid cell colonies in 600 wells to be obtained from the first fusion and 968 colonies in 1700 wells from the second. One of the colonies formed by the first fusion and four colonies from the second fusion produced monoclonal antibodies to angiotensinogen detected by both the RIA and the solid phase assay. All these antibodies were IgG, and their isotypes were the following: IgGl-Clones 77 and 138, IgG2a-Clones 136 and 140, IgG2b-Clone 134.

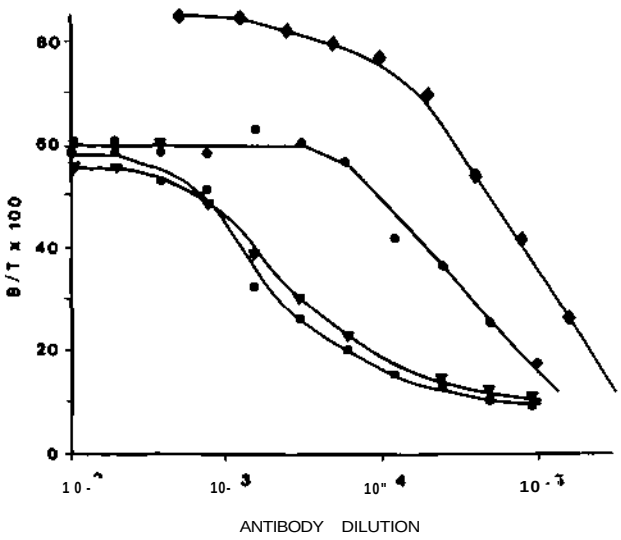

FIGURE 1. *Binding of different monoclonal and potyclonal antibodies to ^{125}I pure angiotensinogen. The percentage of binding is indicated on the ordinate. The dilutions ofascitesfor Clones 77 and 138, of concentrated (20 times) culture supernatant for Clone 136, and of serum for polyclonal antibody are indicated on the abcissa. The different curves represent the following antibodies: •———• monoclonal 77; •———• monoclonal 136; •———• monoclonal 138; •———• polyclonal.*

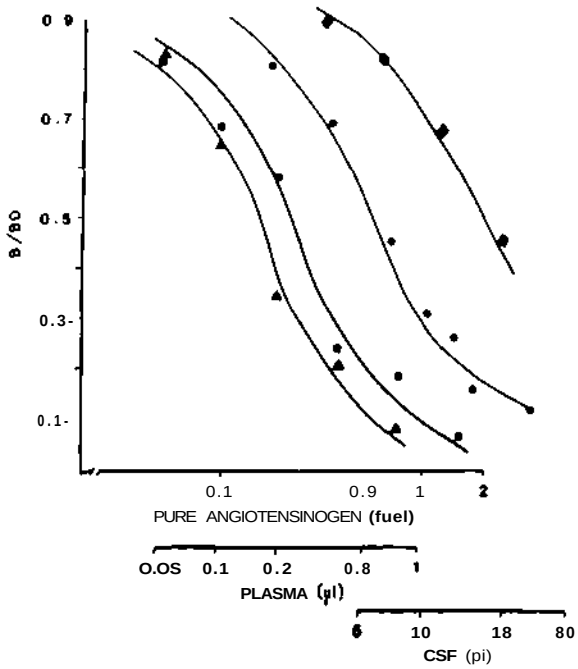

FIGURE 2. *Curves of displacement of ^{ns}l-angiotensinogen by pure angiotensinogen (•———*), plasma angiotensinogen (M———*), cerebrospinal fluid (CSF) angiotensinogen (•———+), and plasma des-angiotensin I-angiotensinogen (A———A) with the use of monoclonal Antibody 136. The percentage of displacement (BIBo) is indicated on the ordinate, and the various concentrations of pure angiotensinogen or volumes of plasma, plasma hydrolyzed by renin, or CSF appear on the abcissa.*

Figure 1 shows the binding of these antibodies to ^{12i}I-angiotensinogen. For Clones 134 and 140, binding was too low for further investigation, as their respective binding maxima were 20% and 30%, and the final ascites dilutions for 50% binding, 1/20 and 1/40. The three other antibodies bound satisfactorily to the tracer, and 50% binding was obtained for final dilutions of 1/35000 for Clone 77, and 1/3500 for Clones 136 and 138. The maximum binding for each of these three antibodies was about 60%, but it rose to 80% when the three antibodies were mixed. For all other procedures, the antibodies were used at a final dilution that bound 50% of the tracer.

Figure 2 shows the displacement curve obtained with pure angiotensinogen for the monoclonal Antibody 136. The sensitivity of the assay with this antibody was 0.2 pmol of angiotensinogen. For both plasma and CSF, dilution curves were parallel to the standard curve. Similar results were obtained with Clones 77 and 138, but their sensitivity was different: 2 pmol for Antibody 136, and 10 pmol for Antibody 138, compared to 5 fmol for polyclonal antibody. The sensitivity of Antibody 138 was too low for it to be tested with CSF angiotensinogen, but adequate for the others. In addition, all three antibodies recognized plasma angiotensinogen and des-angiotensin I-angiotensinogen equally, since their dilution curves were parallel.

Examination of the respective cross-reactivities of these antibodies with different peptides derived from angiotensinogen and with angiotensinogen from other species revealed no cross-reaction, except for mouse angiotensinogen which was recognized by Clone 136 (1%) and Clone 138 (25%, Table 1).

The association constants of the different antibodies varied. The polyclonal antibody exhibited a very high

TABLE 1. *Cross-Reactivity of Three Monoclonal Antibodies Against Rat Angiotensinogen Compared to the Cross-Reactivity of the Potyclonal Antiserum*

	Monoclonal antibodies			Polyclonal antiserum (Bouhnik et al. 1982)
	77	136	138	
Angiotensin I	0	0	0	0
Angiotensin II	0	0	0	0
Tetradecapeptide	0	0	0	0
Human plasma angiotensinogen	0	0	0	0
Monkey plasma angiotensinogen	0	0	0	0
Rabbit plasma angiotensinogen	0	0	0	0
Calf plasma angiotensinogen	0	0	0	0
Mouse plasma angiotensinogen	0	1	25	0.05
Rat plasma des-angiotensin I-angiotensinogen	100	100	100	100

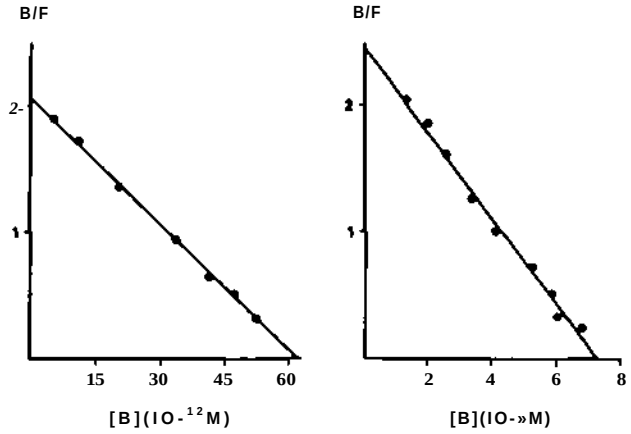

FIGURE 3. *Scatchard curves for the polyclonal antibody (left) and for monoclonal Antibody 136 (right). The amount of pure bound angiotensinogen [BJ is indicated on the abcissa, and the ratio of bound to free angiotensinogen (B/F) is shown on the ordinate.*

affinity for angiotensinogen (K_d = 3 x 10^{-13} M), whereas the respective K_d of the monoclonal antibodies were 3.8 x 10-'°M for Antibody 136, 7.5 x 10^{-9} M for Antibody 77 and 2.5 x 10^{-8} M for Antibody 138. The Scatchard curves were linear for both the polyclonal and monoclonal antibodies (Figure 3).

Inhibition of the renin-angiotensinogen reaction was tested in vitro and compared to the inhibition obtained with the polyclonal antiserum (Figure 4). This serum gave 50% inhibition with 1.5 *fig* of immunoglobulin, but no inhibition was observed with 1.5 */xg* of each monoclonal antibody separately or with the three antibodies mixed together. A 15% inhibition was obtained with a concentration of monoclonal antibody 10 times greater than the concentration of polyclonal antibody

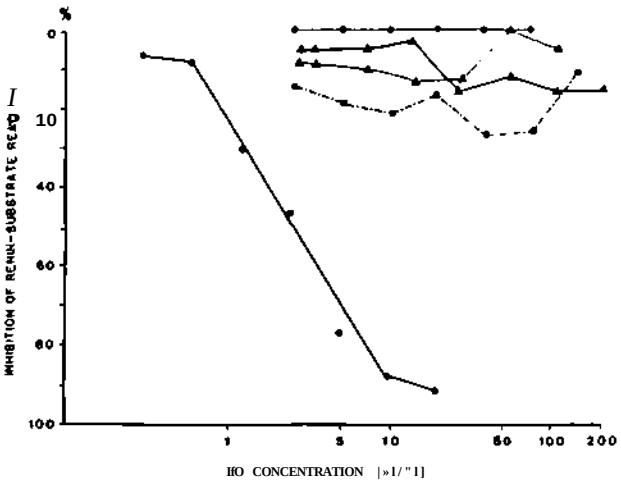

FIGURE 4. *Inhibition of the renin-angiotensinogen reaction. The percentage of inhibition is shown on the ordinate. The amounts of gammaglobulin are indicated on the abcissa for polyclonal antiserum (•————•), for monoclonal Antibodies 77 (•—•), 136* f A—AJ *and 138* (A—A), *and for an equtmolar mixture of the three monoclonal antibodies* (•—*).

giving 100% inhibition. Even though the inhibition increased slightly (20%) when the three antibodies were mixed, it was not dose-dependent, and therefore it could not be considered specific.

Discussion

As two fusions resulted in only three stable monoclonal antibodies to rat angiotensinogen, antigenicity of rat angiotensinogen was low in mice. Antibodies were screened by their capacity to bind pure iodinated angiotensinogen or by solid phase assay. The first test is simple and reproducible and has the advantage of requiring only moderate amounts of pure angiotensinogen, but it has its limitations. Modifications of the angiotensinogen molecule can occur during iodination and lead to underestimation of the number of positive antibodies. Because no additional positive hybrids were detected by the solid phase assay, the radioiodination probably has no adverse effects on angiotensinogen. Inhibition of the renin-angiotensinogen reaction was not used as a screening test, so that an antibody directed against this particular epitope of the molecule may not have been detected by the two radioimmunological tests.

The titratfon of the monoclonal antibodies showed that the maximum binding of monoclonal antibodies with iodinated angiotensinogen did not exceed 60%. This low level may be due either to the low affinity of these antibodies for labeled angiotensinogen or to a problem in achieving complete PEG precipitation of the smaller antigen-antibody complex. The latter explanation is supported by the rise observed in maximum binding when the three monoclonal antibodies were mixed together, thus increasing the size of the antigen-antibody complex. If this assumption is correct, it can be inferred, as a corollary, that each of these antibodies recognizes a different determinant of the angiotensinogen molecule. The different degrees of cross-reactivity observed with mouse angiotensinogen for each monoclonal antibody is also another argument for independent epitopes for the antibodies.

As with the polyclonal antibody,[1] no cross-reaction with monoclonal antibodies was observed for angiotensinogen from species other than rat, with the exception of mouse plasma, which indicates a strong species specificity for this molecule.

Monoclonal antibodies displayed lower titers, sensitivities, and association constants than polyclonal antiserum. For all three monoclonal antibodies, we observed a relationship between the sensitivity and dissociation constant (0.2, 2, 10pmoland3.8 x 10^{-10} M,7.5 x 10-'M, and 2.5 x 10^{-8} M for monoclonal antibodies 136, 77, and 138 respectively), but none between these parameters and the 50% binding of tracer.

Support for the argument that each monoclonal antibody recognizes an identical, nonmodified epitope on the four angiotensinogen molecules tested is provided by the fact that the dilution curves for pure angiotensinogen, plasma, and CSF angiotensinogen and plasma des-angiotensin I-angiotensinogen were parallel.

The identical degree of recognition of plasma and CSF angiotensinogen displayed by two different monoclonal antibodies confirmed that these two molecules, synthesized either in the liver[12] or in the brain,[13] are immunologically identical, as suggested by a previous study[14] that used polyclonal antiserum.

Moreover, all three monoclonal antibodies recognized angiotensinogen as well as its metabolic products (des-angiotensin I-angiotensinogen) or its precursors.[12] This result and the absence of cross-reactions with angiotensin I, II, or tetradecapeptide indicate that the epitope on the angiotensinogen molecule recognized by each antibody is distant from the NH_2 terminal region. This conclusion is supported by the failure of the monoclonal antibodies to inhibit the renin-angiotensinogen enzymatic reaction. The inhibition obtained with the polyclonal antiserum may be due either to the presence of an antibody that recognizes the NH_2 terminal epitope or to steric hindrance by many different antibodies on the angiotensinogen molecule, which prevents the renin enzymatic action. When the three monoclonal antibodies were associated, the 20% inhibition observed was difficult to explain, but was not dose-dependent and was therefore nonspecific.

These preliminary results do not exclude the possibility of eventually obtaining a monoclonal antibody that will recognize the NH_2 terminal epitope and inhibit the enzymatic reaction. For this to happen, however, the effectiveness of the immunization and fusion will have to be drastically increased. Use of such a monoclonal antibody could be very helpful to in vivo study of the physiological role of angiotensinogen in the renin-angiotensin system and in the regulation of blood pressure. The first results with polyclonal antisera were promising.[2]

In conclusion, the affinity characteristic of the monoclonal antibodies tested is positive for their use in immunopurification of rat angiotensinogen, which is at present a six-step procedure. Further, the specificity and affinity of these antibodies will permit the study by immunocytochemical techniques of the different locations of angiotensinogen, particularly in the central nervous system.

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