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MONOCLONAL ANTIBODIES TO ANGIOTENSIN II

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Three hybridomas producing monoclonal antibodies to angiotensin II were generated using somatic cell fusion techniques. Dissociation constants, determined by Scatchard analysis of radioimmunoassay data, were 9.3 x 10^{-10} M, 1.5 x 10^{-9} M and 1.8 x 10^{-8} M for antibodies KAA8, ICH2 and ICA10, respectively. The antibodies exhibited various degrees of cross-reactivity towards peptide metabolites of angiotensin II, but minimal cross-reactivity towards antagonists created by substituting the hormone at both the amino and carboxy terminus. In contrast to a rabbit polyclonal angiotensin II antibody preparation, the three monoclonal antibodies competed effectively with angiotensin receptors on rat adrenal cortical microsomes for hormone binding. These antibodies may have broad utility for a number of applications in angiotensin II research.

Angiotensin II (AII), an octapeptide with potent vasoconstrictor properties, is the active hormone of the renin-angiotensin system (1,2). It is formed by cleavage of a dipeptide from the carboxy-terminus of angiotensin I (AI), its inactive precursor. We report here the development of three hybridoma cell lines that secrete antibody to AII. These antibodies were compared with a polyclonal AII antiserum for their affinity, specificity, and ability to compete with AII receptors on rat adrenal cortical microsomes for hormone binding. The results suggest that these monoclonal antibodies have a number of potential applications for AII research including immunoassays, physiological studies and structural-functional studies.

MATERIALS AND METHODS

Source of peptides. AII (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), AI, AIII, [Sar^1,Ala^8]AII, and [Sar^1,Thr^8]AII were from Peninsula Labs (Belmont, CA). All other peptides were from Bachem (Torrance, CA) except for [Sar^1]AII which was synthesized by A. T. Chiu.

Immunizations. AII was coupled to keyhole limpet hemocyanin (Calbiochem-Behring Corporation, San Diego, CA) using 1-ethyl-3-(3-dimethyl-aminopropyl)

Abbreviations used in this paper: AII, angiotensin II; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay.

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carbodiimide (Bio-Rad Laboratories, Richmond, CA) as described by Freedlender and Goodfriend (3). Adult, female Balb/c mice were primed intraperitoneally with 25 ug of the conjugated hormone in aluminum potassium sulfate (alum). Intraperitoneal boosts of the conjugate were repeated at three week intervals. Antibody titers of 1:1000, as determined by the AII ELISA, were detectable in serum samples three days after the first boost. Fusions were performed 3 days after either the second or third boost.

Cell Fusion. Cells from spleens were fused with P3-X63-Ag8.653 myeloma cell at a 2:1 ratio as described by Oi and Herzenberg (4). PEG 1500 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was the fusing agent. Cells were distributed into 96-well microtiter plates in Iscove's Modified Dulbecco's Medium (IMDM), containing 15% fetal bovine serum and hypoxanthine, aminopterin, and thymidine (HAT medium). Hybridoma growth was observed within 1 to 2 weeks. Selected hybridomas were cloned by limiting dilution as described by Oi and Herzenberg (4).

Screening for antibody-producing clones. Culture supernatants were screened for specific antibody production by an ELISA using microtiter plates coated with AII. The bound immunoglobulins were quantified with sheep anti-mouse ($F(a_b')2$ linked to B-galactosidase (Amersham, Arlington Heights, IL).

Liquid phase radioimmunoassay. Those hybridomas displaying substantial activity in the ELISA (greater than three times the value observed with supernatant from P3 myeloma cells) were tested in the liquid phase RIA using the procedure of Freedlender and Goodfriend (3). [^{125}I]-AII was purchased from New England Nuclear.

Characterization of monoclonal antibodies. The isotype of each monoclonal antibody was determined by Ouchterlony gel diffusion using a mouse isotyping kit (Boehringer Mannheim, Indianapolis, IN). Antibody cross-reactivities were determined by RIA in the presence of varying concentrations of AII agonists and antagonists. Cross-reactivities were computed by the method of Abraham (5). Dissociation constants were determined from Scatchard analysis of the AII inhibition curves (6). Antibody from culture supernatants was purified by affinity chromatography on protein A-Sepharose CL (Pharmacia, Piscataway, NJ) as described by Ey and colleagues (7).

Polyclonal AII serum. Rabbit antiserum to AII was purchased from New England Nuclear. The serum was characterized by the RIA as described above. Immunoglobulins were purified by passage over a protein-A column. Affinity purified rabbit and mouse immunoglobulins were purchased from Cooper Biomedical (Malvern, PA).

Effect of antibodies on AII binding to its receptor. AII receptor-containing rat adrenal cortical microsomes were prepared as described (8). Various concentrations of the purified antibodies were incubated in polystyrene tubes with 20 ug of freshly prepared microsomes in 50 mM Tris buffer at pH 7.2 containing 5 mM $MgCl_2$.6H O, 0.25% bovine serum albumin and 2 nM ^{3}H-AII (27.8 mCi/mmol, New England Nuclear). After 60 min at 25°C, 3 ml of ice-cold Tris buffer were added to each assay tube and the bound and free radioactivity separated by filtration through glass micro-fibre filter strips (Reeves Angle 934 AH). The filters were dried and the trapped radioactivity determined in a Packard PRIAS scintillation counter. Data was displayed as specific binding, that is the binding displaceable by 10 uM unlabeled AII. The inhibitory concentration of antibody that gave 50% displacement of the total specific bound ligand (IC_{50}) was determined from the displacement curves.

RESULTS

Forty-two anti-AII hybridomas were identified by ELISA from a fusion utilizing spleen cells from a mouse that had been immunized three times with

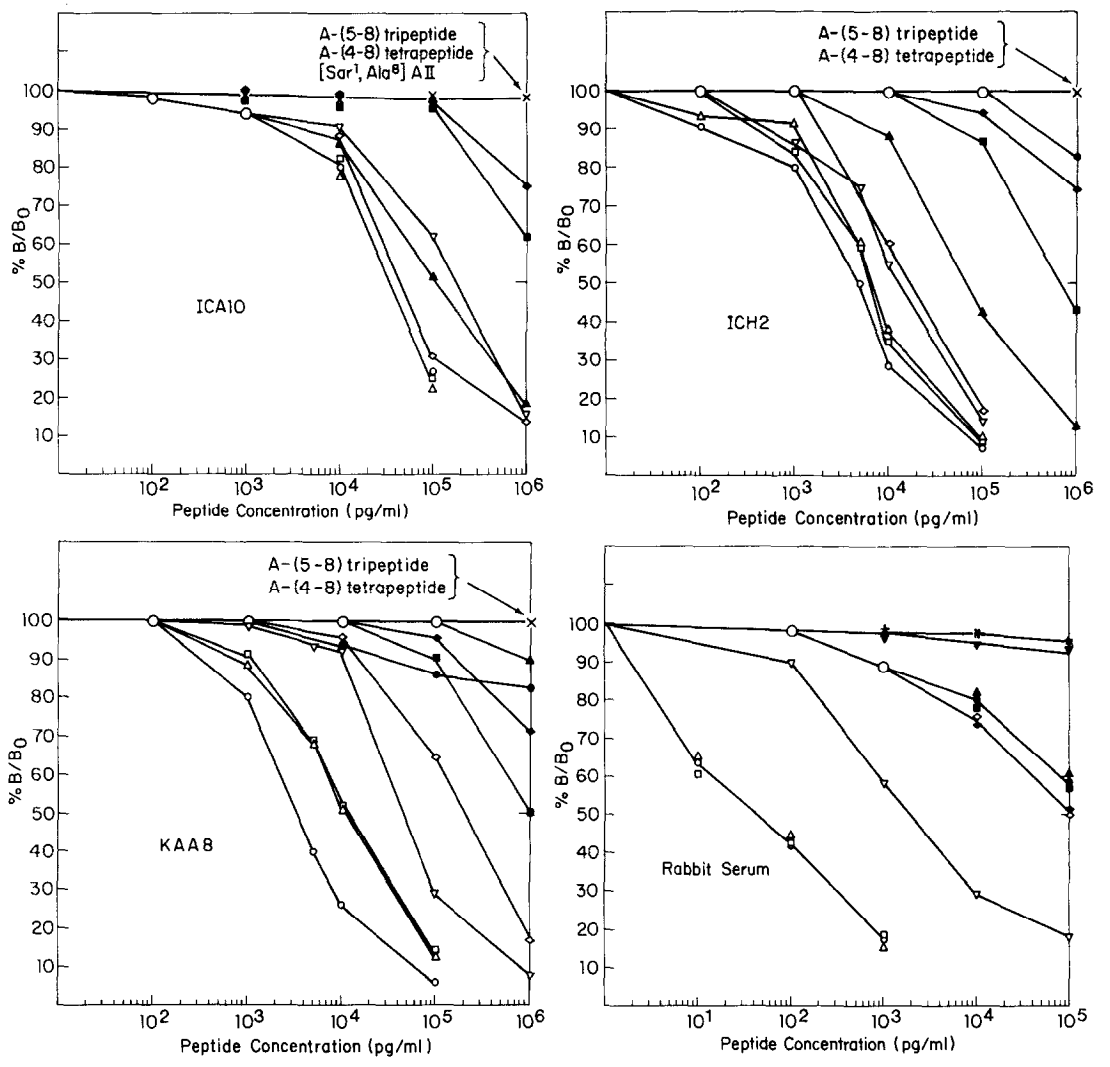

Figure 1. Inhibition of binding of ^{125}I-AII to monoclonal antibodies ICA10, ICH2 and KAA8, and to a polyclonal AII antiserum. Competing ligands include: (○) A-(1-8) octapeptide (AII), (▽) A-(1-10) decapeptide (AI), (□) A-(2-8) heptapeptide (AIII), (◇) A-(3-8) hexapeptide, (▲) A-(4-8) pentapeptide, (▼) A-(5-8) tetrapeptide, (+) A-(6-8) tripeptide, (△) [Sar^1]AII, (●) [Sar^1,Ala^8]AII, (■) [Sar^1,Cys^8]AII, and (◆) [Sar^1,Thr^8]AII.

AII-KLH. Only two of the hybridomas contained antibody which was positive in the RIA. These two hybridomas were designated ICH2 and ICA10. A third positive hybridoma, KAA8, was isolated from a subsequent fusion utilizing spleen cells from a mouse that had been immunized four times with AII-KLH. All three hybridomas were subcloned by limiting dilution and produced antibody of the IgG1 subclass.

One hundred microliters of culture supernatant from ICA10, fifty from ICH2 and ten from KAA8 bound approximately 50% of 10 pg [^{125}I]-AII in the RIA. Comparable binding was observed with 100 ul of a rabbit polyclonal AII

Table 1. Cross-reactivity[a] of AII antibodies with peptide agonists and antagonists of AII

Peptide	% Cross-Reactivity			
	ICA10	ICH2	KAA8	Polyclonal AII Serum
A-(1-8) octapeptide (AII)	100	100	100	100
A-(6-8) tripeptide	nc[b]	nc	nc	nc
A-(5-8) tetrapeptide	nc	nc	nc	nc
A-(4-8) pentapeptide	26	7	nc	0.03
A-(3-8) hexapeptide	66	28	2	0.06
A-(2-8) heptapeptide (AIII)	100	77	32	99
A-(1-10) decapeptide (AI)	20	36	7	6
[Sar^1]AII	100	77	32	100
[Sar^1,Ala^8]AII	nc	nc	nc	0.03
[Sar^1,Cys^8]AII	0.5	0.7	0.32	0.03
[Sar^1,Thr^8]AII	0.5	nc	nc	0.06
Kd	1.8×10^{-8}	1.5×10^{-9}	9.3×10^{-10}	1×10^{-10}

[a] Cross reactivity (CR) values were obtained from the equation: CR=pg of AII (1-8) required to inhibit 50% binding of [^{125}I]-AII to antibody/pg of peptide required to inhibit 50% binding x 100.

[b] No cross-reaction (<0.01%).

serum at a 1:5000 dilution. These concentrations were then utilized in RIA studies to examine the specificity of the antibodies for AII and their cross-reactivities with various AII agonists and antagonists (Fig. 1). Relative cross-reactivities calculated from Figure 1 are shown in Table 1 with the reactivity of each antibody with AII taken as 100%.

Scatchard analysis of the AII inhibition curves revealed linear plots for the three monoclonal antibodies (Figure 2). In contrast, a curvilinear plot was observed with the rabbit antiserum reflecting the polyclonal nature of the antibody. Dissociation constants of 1.8×10^{-8} M, 1.5×10^{-9} M and 9.3×10^{-10} M were calculated for antibodies ICA10, ICH2 and KAA8. A Kd of 1.0×10^{-10} M was calculated for the polyclonal serum.

Different dilutions of the protein A-purified antibodies were tested for their ability to inhibit the binding of ^{3}H-AII to its receptors on rat adrenal cortical microsomes. As shown in Figure 3, the monoclonal antibodies effectively inhibited ^{3}H-AII binding in this assay. IC_{50}s were estimated at 1.8×10^{-8} M for ICA10, 1×10^{-8} M for ICH2 and 7.3×10^{-9} M for KAA8. The polyclonal antibody preparation was not as effective with the highest concentration tested (8×10^{-7} M) inhibiting binding by only 40%. Control mouse and rabbit immunoglobulin had no effect on AII binding. In this system, IC_{50}s for AII and its antagonist [Sar^1,Ala^8]AII (saralasin) were calculated at 4×10^{-9} M and 3×10^{-9} M, respectively.

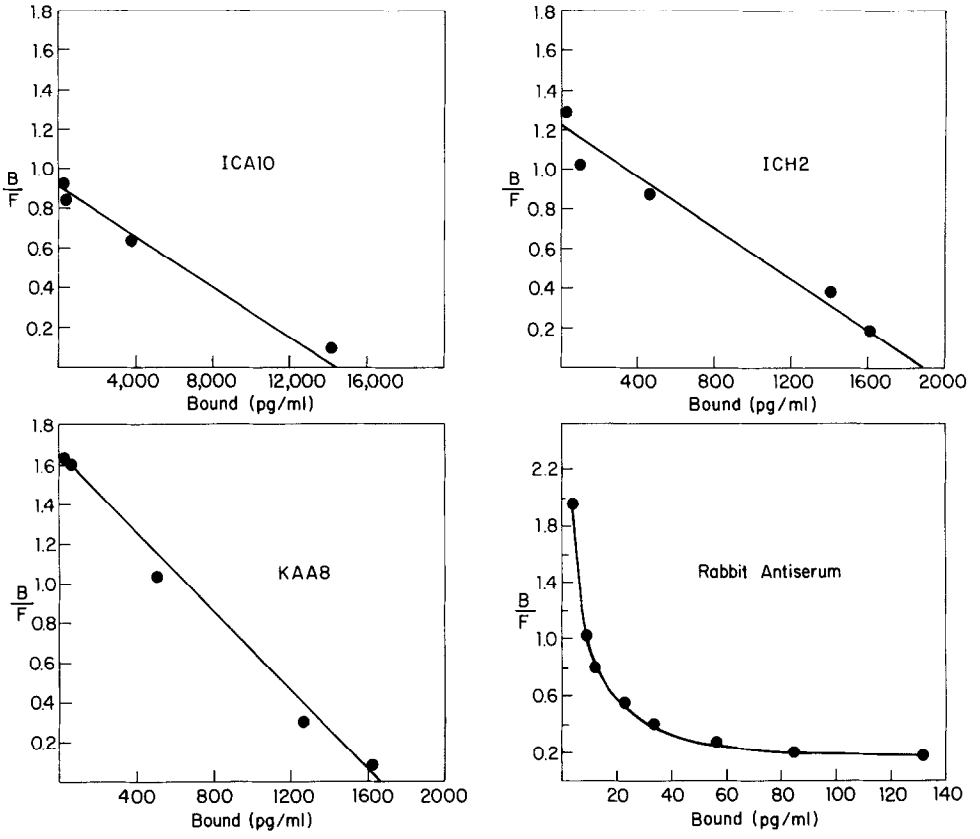

Figure 2. Scatchard analysis of AII inhibition curves for ICA10, ICH2, KAA8 and the rabbit antiserum to AII.

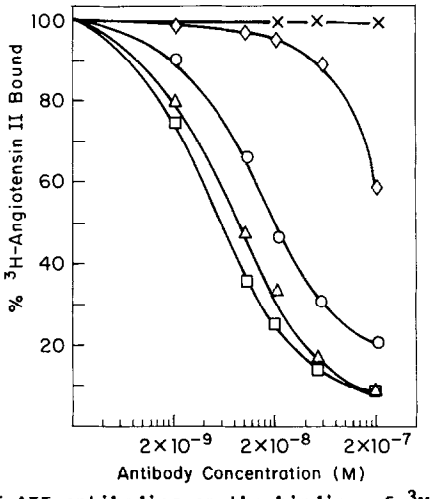

Figure 3. Effects of AII antibodies on the binding of ^{3}H-AII to its receptors on rat adrenal cortical microsomes. Purified monoclonal antibodies and control mouse and rabbit immunoglobulins were resuspended to a concentration of 150 µg/ml. Immunoglobulins purified from the rabbit AII antiserum and control rabbit immunoglobulins were resuspended to a concentration of 600 µg/ml. Different concentrations of these antibodies were then tested in the ^{3}H-AII binding assay. Data is expressed as a percentage of ^{3}H-AII binding relative to that observed in the absence of antibody. (○) ICA10, (△) ICH2, (□) KAA8 (◇) immunoglobulins from the polyclonal AII serum (×) control rabbit and mouse immunoglobulins.

DISCUSSION

By using AII coupled to hemocyanin as an immunogen, we have successfully generated three hybridomas that secrete antibodies to AII. Immunization with AII-protein conjugates almost invariably results in antisera that recognize the carboxy terminus of the molecule, and are therefore incapable of distinguishing AII from its (2-8) heptapeptide metabolite, AIII (9). Consistent with this pattern, ICA10, ICH2 and the rabbit polyclonal sera displayed high cross-reactivity towards AIII in the RIA. However, KAA8 displayed considerably less cross-reactivity (32%) suggesting this antibody is not as specific for a carboxy terminus epitope as are the others. This antibody may be particularly useful for measuring AII levels in serum samples free from the usual interfering effects of AIII.

All the antibodies except KAA8 exhibited full cross-reactivity with AII substituted at the amino terminus with sarcosine ([Sar^1]AII). This observation is consistent with the carboxy terminus specificity of these antibodies in comparison with KAA8, which exhibited only 35% cross reactivity with [Sar^1]AII. None of the antibodies in this study displayed substantial cross-reactivity with antagonists of AII created by substituting at both the carboxy and amino terminus ([Sar^1,Ala^8]AII, [Sar^1,Cys^8]AII and [Sar^1,Thr^8]AII).

All three monoclonal antibodies competed effectively with the AII receptor on rat adrenal cortical membranes for hormone binding. This contrasts with the much smaller inhibitory effect of the immunoglobulins from the polyclonal serum. These results suggest that the monoclonal antibodies bind at or close to the epitope on the hormone recognized by the receptor, while only a certain population of the antibodies in the polyclonal antiserum bind at this site(s). Sen and coworkers (10) have previously demonstrated the presence of determinants of AII that do not interact with the receptor.

Monoclonal antibodies to AII have been described by Haber and coworkers (11), and more recently by Couraud (12). The former group raised one antibody which displayed a relatively low affinity for AII (0.3×10^7 M^{-1}), and which resembled polyclonal AII sera in its specificity for the hormone's carboxy terminus. One of the antibodies generated by Couraud, designated A22, resembles KAA8 in its high affinity for AII, its low affinity for AII antagonists, and because it recognizes an epitope which seems to overlap the amino terminal moiety of AII. Neither group reported on the ability of their antibodies to compete with the AII receptor for hormone binding.

In summary, these monoclonal antibodies may be well suited for a number of applications in AII research including immunoassays, immunohistochemical studies and structural-functional studies of the hormone.

Furthermore, the ability of these antibodies to compete with receptors for hormone binding suggests an additional application: functional antagonists of AII in physiological studies. Since AII is the effector molecule of the renin-angiotensin system, an important regulator of blood pressure and extracellular fluid homeostasis (1-2), specific antagonists of the hormone would represent useful physiological tools in studying the development and maintenance of AII-dependent hypertension. Currently, such studies with these antibodies are in progress in our laboratory.

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REFERENCES

1. Oparil, S., and Haber, E. (1974) N. Engl. J. Med. 291, 389-401.
2. Skeggs, L.T. (1983) In Hypertension and the Angiotensin System (A.E. Doyle and A.G. Bearn, eds.) pp. 31-45. Raven Press, New York, NY.
3. Freelender, A.F. and Goodfriend, T.L. (1979) In Methods of Hormone Radioimmunoassay (B.M. Jaffe and H.R. Behrman, eds.) pp. 889-907. Academic Press, New York, NY.
4. Oi, U.T., and Herzenberg, A. (1980) In Selected Methods in Cellular Immunology (B.B. Mishell and S.M. Shiigi, eds.) pp. 351-372. W.H. Freeman and Co., San Francisco, CA.
5. Abraham, G.W. (1969) J. Clin. Endocrinol. Metab. 29, 886-895.
6. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-668.
7. Ey, P.L., Prowse, S.J., and Jenkin, C.R. (1978) Immunochemistry 15, 429-437.
8. Gunther, S., Gimbrone, M.A., and Alexander, R.W. (1980) Circ. Res. 47, 278-285.
9. Nussberger, J., Matsueda, R., and Haber, E. (1983) J. Immunol. Methods 56, 85-96.
10. Sen, I., Bull, H.G., and Soffer, R.L. (1984) Proc. Natl. Acad. Sci. USA 81, 1679-1684.
11. Nussberger, J., Mudgett-Hunter, M., Matsueda, G., and Haber, E. (1984) Hybridoma 3, 373-376.
12. Couraud, P. (1986) J. Immunol. 136, 3365-3370.