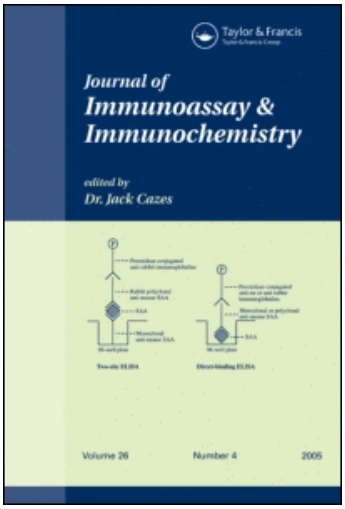

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Odya, Charles E. , Carlin, Robert J. , Yapa, Ramani D. and Soltani-Tehrani, Bahram(1993) 'Immunoassays for des-Arg^9-Bradykinin', Journal of Immunoassay and Immunochemistry, 14: 4, 227 — 240
To link to this Article: DOI: 10.1080/15321819308019852
URL: http://dx.doi.org/10.1080/15321819308019852

JOURNAL OF IMMUNOASSAY, 14(4), 227-240 (1993)

IMMUNOASSAYS FOR DES-ARG^9-BRADYKININ

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ABSTRACT

Splenocytes from a female, BALB/c mouse immunized with bradykinin conjugated to ovalbumin with toluene diisocyanate were fused with mouse myeloma cells, X63/Ag8.653, using polyethylene glycol. Seventy-nine hybridomas were identified by ELISA to be making kinin reactive antibodies. In preliminary specificity studies it was determined that all of these hybridomas were producing antibodies more reactive with des-Arg^9-bradykinin than with bradykinin. ELISAs were developed with the five clones that displayed the highest affinities for des-Arg^9-bradykinin. Radioimmunoassays were developed for 3 of these 5 clones as well as with 5 monoclonal antibodies previously described (Odya and Lee 1990). The most sensitive des-Arg^9-bradykinin assay developed was a radioimmunoassay in which carboxypeptidase B-treated [Tyr^5]-bradykinin was the labeled antigen, clone OLNBK-5 was the antibody, and dextran-coated charcoal was used to separate bound from free radioactivity. The concentration of des-Arg^9-bradykinin that inhibited 50% of the radioactive peptide binding was 0.08 $\pm$ 0.03 nM. The relative specificity of this assay (des-Arg^9-bradykinin = 100%) was: 29% bradykinin and about 1% with each of the following: lysyl-bradykinin, methionyl-lysyl-bradykinin, des-Arg^1-bradykinin and des-Phe^8-Arg^9-bradykinin. (KEY WORDS: Kinins, Monoclonal antibodies, Immunoassays).

INTRODUCTION

Kinins are peptides released from kininogens by kallikreins (1). Kinins have been implicated as being involved in blood pressure regulation (2), renal function

(3) and as mediators of inflammation (4). Receptors selective for des-Arg^9-bradykinin have been termed B1 bradykinin receptors, while those preferring bradykinin over des-Arg^9-bradykinin have been termed B2 bradykinin receptors (5). The physiological and/or pathological importance of B1 bradykinin receptors in mediating effects of kinins is not known. However, blood levels of des-Arg^9-bradykinin have been reported to be ten times higher than bradykinin in normal humans (6). Radioimmunoassays for des-Arg^9-bradykinin to date have used polyclonal antibodies (7, 8).

The purpose of the present study was 1. to prepare monoclonal kinin antibodies and evaluate their usefulness in immunoassays for des-Arg^9-bradykinin and 2. to determine whether previously described monoclonal kinin antibodies (9), when used in radioimmunoassays, would yield assays more sensitive for des-Arg^9-bradykinin than those previously described (7, 8). Sensitive immunoassays for des-Arg^9-bradykinin utilizing monoclonal antibodies could make assays for this B1 bradykinin receptor agonist more widely available. The application of these assays to measurements of des-Arg^9-bradykinin in biological fluids should help to assess the involvement of this kinin in physiological and pathological processes.

MATERIALS AND METHODS

Materials

Monoclonal kinin antibodies, OLNBK-1, OLNBK-2, OLNBK-3, OLNBK-4 and OLNBK-5, reagents for performing fusions and ELISAs were obtained as previously described (9). The sources of the materials used to prepare the iodinated kinins and the bradykinin-ovalbumin conjugate were as described previously (6). BALB/c mice were purchased from Harlan, Indianapolis, IN. X63/Ag8.653 myeloma

cells were obtained from the Indiana University biology tissue culture facility, Bloomington, IN.

Methods

Immunization and Fusion

Bradykinin was coupled to ovalbumin following the procedure of Talamo et al. (10). A 3 month old, female BALB/c mouse was immunized subcutaneously at 6 sites with a total of 0.14 mg of bradykinin-ovalbumin conjugate suspended in RIBI adjuvant. Five booster injections of amounts comparable to the initial immunization were administered intraperitoneally at 2 week intervals. Three days after the last immunization the mouse was sacrificed and the fusion performed and screened as previously described (9). However, X63/Ag8.653 myeloma cells were used instead of SP2/o cells.

Immunoassays

ELISAs (9) and radioimmunoassays (7) were performed according to previously described procedures. Hybridoma tissue culture supernatants were the source of antibodies used in the present experiments. Lysyl-bradykinin and des-Arg^9-bradykinin were coupled to bovine serum albumin using glutaraldehyde as described previously for bradykinin (9).

Dilutions of hybridoma supernatants were incubated in ELISA plates coated with varying dilutions of each of the three kinin-BSA conjugates, lysyl-bradykinin-BSA, bradykinin-BSA, and des-Arg^9-bradykinin-BSA, in order to determine which conjugate yielded the greatest dilution of hybridoma supernatant to give an A_{405} between 0.5 and 1.0. Similarly dilutions of hybridoma supernatants were incubated

with ^{125}I-labeled kinin analogues, Tyr^1-kallidin, Tyr^5-bradykinin, and Tyr^8-bradykinin, to determine which radioactive antigen permitted the greatest dilution of antibody that would bind 30% of the radioactive peptide incubated with it.

Data analysis

Results recorded in this report are the means $\pm$ S.D. of at least three experiments each of which was performed in triplicate.

RESULTS

Of the 2880 wells plated with fused cells, 2199 or 76% displayed hybridoma growth. Seventy-nine or 3.6% of these were determined by ELISA to be making kinin reactive antibodies. All of the hybridomas were determined in preliminary specificity experiments to be making antibodies more reactive with des-Arg^9-bradykinin than bradykinin. The 5 antibodies that displayed the highest affinities for des-Arg^9-bradykinin were selected for further characterization. Each of these antibodies displayed greater reactivity with ELISA plates coated with either lysyl-bradykinin-BSA or bradykinin-BSA than with des-Arg^9-bradykinin-BSA (data not shown). Since signals were slightly greater with lysyl-bradykinin-BSA coated plates than bradykinin-BSA coated plates, lysyl-bradykinin-BSA was used to coat plates used to generate the data recorded in Table 1. For the sake of comparison, ELISA data for five monoclonal kinin antibodies previously described by this laboratory (Odya and Lee 1990) are included in Table 1. The new monoclonal antibodies all have lesser affinities for des-Arg^9-bradykinin than do the previously characterized antibodies. However, OCMSP-1, OCMSP-2, and OCMSP-3 displayed greater selectivity for des-Arg^9-bradykinin over bradykinin than do the previously characterized antibodies.

All of the antibodies listed in Table 1 displayed greater reactivity with carboxypeptidase B-treated (CB) derivatives of mono ^{125}I-labeled derivatives of Tyr^1-kallidin (T1K) Tyr^5-bradykinin (T5BK), and Tyr^8-bradykinin (T8BK), when tested in radioimmunoassays than with the intact derivatives. In general, antibodies reacted about equally well with CBT5BK and CBT8BK but to a much lesser degree with CBT1K (data not shown). Recorded in Table 2 are affinity and specificity data obtained for the antibodies in radioimmunoassays. Data were not obtained for OCMSP-4 and OCMSP-5, because these antibodies did not bind the radioactive antigens tightly enough to permit the establishment of radioimmunoassays with them.

Shown in Figure 1 is the des-Arg^9-bradykinin inhibition curve obtained with the most sensitive radioactive antigen-antibody combination, CBT5BK-OLNBK-5. The IC_{50} for des-Arg^9-bradykinin was 0.08 $\pm$ 0.03 nM.

DISCUSSION

In our initial report on the generation of monoclonal kinin antibodies (9) bradykinin had been coupled to carrier proteins using carbodiimide. This coupling agent makes conjugates of bradykinin through its N-terminus and also its C-terminus. Of the 9 monoclonal antibodies obtained in that study, 4 displayed greater reactivity for bradykinin than des-Arg^9-bradykinin. In the present report the coupling agent used, diisocyanate, links bradykinin only through its N-terminus to the carrier protein. Despite the fact that bradykinin was conjugated to ovalbumin, all of the antibodies obtained displayed greater reactivity with des-Arg^9-bradykinin than with bradykinin. This finding leads us to hypothesize that if the antibody desired is one that displays greater reactivity with bradykinin than des-Arg^9-

TABLE 1

Specificities of [Des-Arg^9]-Bradykinin Monoclonal Antibodies by ELISA

Antibody	Kinin Analog					
	-9BK	BK	LBK	MLBK	-1BK	-89BK
OLNBK-1	1.2* ± 0.2 (100%)+	10 ± 0.2 (120%)	29 ± 4 (4.1%)	24 ± 8 (5.0%)	34 ± 8 (3.5%)	3 ± 15 (3.8%)
OLNBK-4	1.6 ± 0.5 (100%)	4.2 ± 0.8 (38%)	88 ± 34 (1.8%)	71 ± 33 (2.2%)	41 ± 12 (3.9%)	37 ± 12 (4.3%)
OLNBK-5	1.7 ± 0.5 (100%)	5.9 ± 2.7 (29%)	121 ± 16 (1.4%)	97 ± 26 (1.8%)	72 ± 28 (2.4%)	81 ± 21 (2.1%)
OLNBK-2	2.7 ± 0.0 (100%)	2.8 ± 0.4 (96%)	46 ± 8 (5.8%)	59 ± 22 (4.6%)	91 ± 11 (3.0%)	78 ± 14 (3.5%)
OLNBK-3	5.3 ± 0.9 (100%)	4.1 ± 1.2 (129%)	97 ± 20 (5.5%)	77 ± 12 (6.9%)	135 ± 28 (3.9%)	22 ± 8 (24%)

OCMSP-1	7.9 ± 1.6 (100%)	73.6 ± 12.0 (11%)	2165 ± 151 (0.4%)	2672 ± 1167 (0.3%)	604 ± 340 (1.3%)	684 ± 212 (1.2%)
OCMSP-2	25 ± 5 (100%)	321 ± 96 (7.8%)	10,011 ± 4318 (0.2%)	9516 ± 3864 (0.3%)	1190 ± 397 (2.1%)	2351 ± 524 (1.1%)
OCMSP-3	38 ± 11 (100%)	409 ± 54 (9.3%)	14,434 ± 2305 (0.3%)	18,864 ± 5354 (0.2%)	1536 ± 448 (2.5%)	2743 ± 631 (1.4%)
OCMSP-4	761 ± 164 (100%)	3144 ± 89 (24%)	5203 ± 2214 (15%)	4559 ± 1746 (17%)	18,758 ± 1568 (4%)	>213,767 (<0.4%)
OCMSP-5	3393 ± 350 (100%)	7483 ± 2368 (45%)	17,600 ± 53,349 (1.9%)	197,020 ± 25,841 (1.7%	28,905 ± 2301 (12%)	>213,767 (<1.6%)

* Values listed are the mean ± standard deviation of 3-4 separate experiments and are the nanomolar concentrations of analogs that inhibit by 50% (IC_{50}) the absorbance obtained when antibody is incubated in the absence of analogs.

+ Numbers in parentheses represent the relative specificity of the antibody. The percentage was obtained by dividing the IC_{50} for -9BK by the IC_{50} for -9BK or the kinin analog in question and then multiplying by 100.

TABLE 2

Specificities of [Des-Arg^9]-Bradykinin Monoclonal Antibodies by Radioimmunoassay

Antibody	Kinin Analog					
	-9BK	BK	LBK	MLBK	-1BK	-89BK
OLNBK-5	0.08* ± 0.03 (100%)+	0.28 ± 0.4 (29%)	5.5 ± 2.4 (1.4%)	6.6 ± 0.9 (1.2%)	15.6 ± 3.3 (0.5%)	5.5 ± 2.0 (1.4%)
OLNBK-4	0.7 ± 0.04 (100%)	0.40 ± 0.03 (68%)	2.1 ± 0.4 (13%)	2.6 ± 0.1 (10%)	4.3 ± 0.5 (6.3%)	6.0 ± 1.3 (4.5%)
OCMSP-2	0.54 ± 0.02 (100%)	1.4 ± 0.2 (38%)	10.9 ± 1.0 (5.0%)	15.0 ± 2.2 (3.6%)	64.6 ± 2.2 (0.8%)	101 ± 55 (0.5%)
OLNBK-2	0.58 ± 0.11 (100%)	1.6 ± 0.5 (36%)	21.2 ± 4.2 (2.7%)	23.7 ± 4.1 (2.4%)	30.6 ± 6.2 (1.9%)	19.2 ± 5.2 (3.0%)

OLNBK-1	0.62 ± 0.05 (100%)	1.4 ± 0.5 (44%)	31.4 ± 3.2 (2.0%)	30.6 ± 2.5 (2.0%)	29.3 ± 3.5 (2.1%)	28.3 ± 4.4 (2.2%)
OLNBK-3	0.70 ± 0.16 (100%)	1.0 ± 0.1 (70%)	8.3 ± 1.1 (8.4%)	12.1 ± 1.5 (5.8%)	17.6 ± 2.7 (4.0%)	4.0 ± 1.0 (18%)
OCMSP-3	3.0 ± 0.2 (100%)	10.5 ± 1.1 (28%)	75.9 ± 10.9 (4.0%)	123 ± 5 (2.4%)	283 ± 38 (1.1%)	355 ± 59 (0.8%)
OCMSP-1	3.3 ± 0.3 (100%)	8.6 ± 2.1 (38%)	58.9 ± 14.7 (5.6%)	74.6 ± 9.6 (4.4%)	591 ± 158 (0.6%)	348 ± 70 (0.9%)

* Values listed are the mean ± standard deviation of 3 separate experiments and are the nanomolar concentrations of analogs that inhibit by 50% the amount of binding of ^{125}I-labeled kinin obtained when incubated with antibody in the absence of unlabeled polypeptide.

+ Numbers in parentheses represent the relative specificity of the antibody. The percentage was obtained by dividing the IC_{50} for -9BK by the IC_{50} for -9BK or the kinin analog in question and then multiplying by 100.

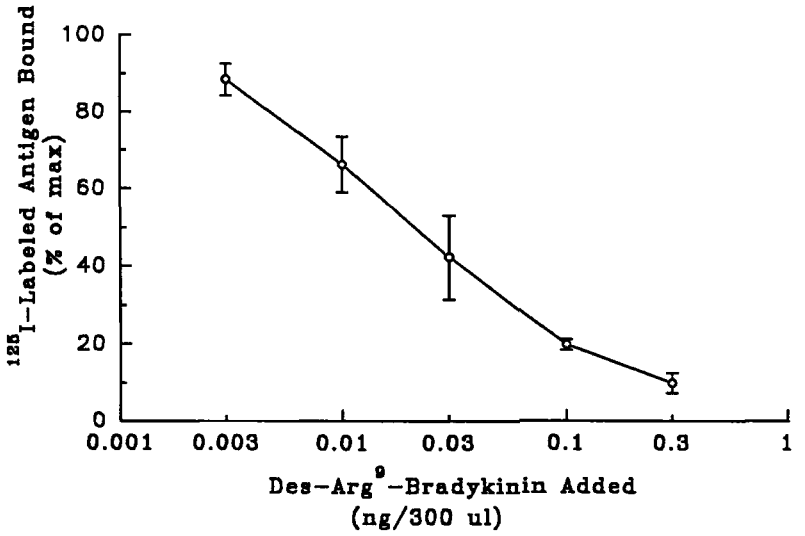

FIGURE 1

Composite of three des-Arg^{9}-bradykinin inhibition curves obtained with OLNBK-5 antibody and CBT5BK as the labeled antigen. Brackets represent one standard deviation from the mean. Incubation and separation were performed as previously described (7).

bradykinin, it would be wise to couple bradykinin through its C-terminus to the carrier protein. Presumably, bradykinin, when coupled to carrier through its N-terminus, is very susceptible to cleavage of its C-terminal arginine. As a result, all of the antibodies obtained when this immunogen is used display greater reactivity with des-Arg^{9}-bradykinin than bradykinin. Further experiments will be required to test the hypothesis.

Although none of the latest monoclonal des-Arg^{9}-bradykinin reactive antibodies were as susceptible in ELISAs to inhibition by des-Arg^{9}-bradykinin (Table 1) as OLNBK-1, OLNBK-2, OLNBK-3, OLNBK-4, and OLNBK-5 previously

described (9), they were in most cases more selective for des-Arg^9-bradykinin than bradykinin.

Three kinin conjugates, lysyl-bradykinin-BSA, bradykinin-BSA and des-Arg^9-bradykinin-BSA were evaluated with kinin antibodies OCMSP-1, -2, -3, -4 and -5 to determine which would result in the most sensitive ELISA. Surprisingly, the lysyl-bradykinin-and bradykinin-BSA conjugates were found to be superior to the des-Arg^9-bradykinin-BSA conjugate for ELISAs despite the fact that the antibodies are more reactive with des-Arg^9-bradykinin than bradykinin. MBK1 (11) a mouse monoclonal antibody even more selective for des-Arg^9-bradykinin than any of the monoclonals this laboratory has produced, also displayed strong reactivity with the lysyl-bradykinin-BSA conjugate in an ELISA (unpublished observation). From these results we conclude that it would not be possible to screen hybridoma supernatants for antibodies specific for a given kinin analog using kinin-BSA conjugates. Apparently the conjugation process leads to an alteration in the peptide conformation such that antibodies, which don't react with the peptide free in solution, now react.

Given the kinin binding specificities and affinities of the monoclonal antibodies (Table 1), it was not surprising to find that all the antibodies preferred the mono-iodinated kinins lacking C-terminal arginines over the intact peptides and that the Tyr^1-kallidin derivative was bound the least. OCMSP-4 and OCMSP-5 which displayed the lowest affinities for kinins in ELISA also did not bind the radioactive antigens tightly enough to evaluate them for radioimmunoassays.

In general, the radioimmunoassays were about an order of magnitude more sensitive than the corresponding ELISAs. However, it is possible that with coating

agents that allow greater dilution of antibodies for ELISAs and with an amplification system greater than that employed in these experiments, it might be possible to develop ELISAs which are comparable in sensitivity to the radioimmunoassays. The overall relative specificities of the antibodies did not change when comparing ELISA (Table 1) and radioimmunoassay (Table 2) results. This is not surprising since specificity should be a property of the antibody and not the assay. Any differences between the assays may be attributed to the fact that the radioimmunoassays (16 hr incubation) are performed under conditions where the reactions come to equilibrium with lower affinity peptides while in the ELISAs (1 hr incubation) this is not the case.

The most sensitive labeled antigen antibody combination, CBT5BK and OLNBK-5 (Fig 1), resulted in a radioimmunoassay which is about 3 times more sensitive for des-Arg^9-bradykinin than that developed previously with a rabbit polyclonal kinin antibody (7). It also displayed a 3 fold greater cross-reactivity with bradykinin 29% vs 8.6%. However, simultaneous immunoassays for bradykinin and des-Arg^9-bradykinin using antibodies highly selective for bradykinin versus des-Arg^9-bradykinin e.g. SBK1 (12) should make it possible to correct immunoreactive des-Arg^9-bradykinin values that may be due to cross-reactivity with bradykinin. The availability of monoclonal antibodies reactive with des-Arg^9-bradykinin should make the availability of immunoassays for this B1-bradykinin receptor agonist more widely available. Application of these assays to measurements of des-Arg^9-bradykinin in samples obtained under normal and pathological states should help to elucidate the importance of this component of the kallikrein-kinin system.

This work was supported by a National Institutes of Health grant HL38833.

ACKNOWLEDGEMENT

We thank Dr. C.-H. Lee and the Naval Medical Research Institute, Bethesda, MD for supplying OLNBK-1, -2, -3, -4, and -5.

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