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ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR KININS USING HIGH-AFFINITY MONOCLONAL KININ ANTIBODIES

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Abstract—Splenocytes from mice immunized either with bradykinin conjugated with carbodiimide to keyhole limpet hemocyanin or ovalbumin were fused using polyethylene glycol with the mouse myeloma cell line SP2/o. Nine monoclonal antibodies reactive with kinins were obtained from two fusions. All of the antibodies were of the IgG_{1k} isotype, except for one, which was an IgG_{2ak}. Based on their reactivities with biologically active kinins and biologically inactive degradation products, the antibodies were separated into three groups. The first group, which had the highest affinities for bradykinin, displayed about equal reactivities for bradykinin and des-Arg^9-bradykinin, but little reactivities for the kinin fragments, des-Arg^1-bradykinin and des-Phe^8-Arg^9-bradykinin, or for lysyl-bradykinin and methionyl-lysyl-bradykinin. The second group was similar to the first except that it showed about a 2.5- to 3.5-fold greater reactivity for des-Arg^9-bradykinin than for bradykinin. The third group, which had the lowest affinities for bradykinin [50% inhibition of antibody binding to an enzyme-linked immunosorbent assay (ELISA) plate occurring with bradykinin concentrations ranging from about 8 to 39 nM], showed little reactivities with des-Arg^1-bradykinin, des-Arg^9-bradykinin and des-Phe^8-Arg^9-bradykinin, but 50–100% cross-reactivities with lysyl-bradykinin and methionyl-lysyl-bradykinin. The useful ranges for bradykinin detection (ng/well, 50 μL assay volume) using the highest affinity antibody in each group in ELISAs were: 0.01 to 0.5, 0.03 to 3, and 0.1 to 3 for groups 1, 2, and 3, respectively.

The kallikrein–kinin system has been implicated as having a role in a number of physiological and pathological conditions [1, 2]. Antagonists have been developed for the known types of kinin receptors, B1 [3] and B2 [4]. However, these antagonists are peptides with relatively low affinities for kinin receptors and may have a short duration of action *in vivo* due to their inactivation by peptidases and/or their removal from the circulation by renal excretion. The purpose of the present study was to prepare antibodies reactive with kinins that could be used in experiments to block the actions of kinins by competing with kinin receptors for the available kinin or that could be used in experiments to elicit anti-idiotypic antibodies that block kinin receptors by cross-reacting with the kinin binding sites of these receptors. Hybridoma technology [5] was applied in the production of kinin antibodies so that an unlimited supply of antibodies would be available for future experiments. This paper describes the production and characterization of monoclonal antibodies reactive with kinins as well as the development of ELISAs† for kinins using these antibodies.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO): EDAC, KLH, BSA, glutaraldehyde, hypoxanthine, aminopterin, thymidine, alkaline phosphatase conjugated goat anti-mouse polyvalent immunoglobulins, goat anti-mouse polyvalent immunoglobulins, *p*-nitrophenyl phosphate disodium, Triton X-100, 2-mercaptoethanol, −9BK, LBK, MLBK, and ovalbumin. Colcemid® (*n*-methyl-*n*-deacetyl-colchicine) and PEG 1500 were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Alkaline phosphatase conjugated to Protein A and mouse isotyping reagents, Mouse Monoab-ID EIA Kit/AP, were purchased from Zymed Laboratories, Inc. (San Francisco, CA). STM and RAS were from Ribi Immuno Chemical Research, Inc. (Hamilton, MT). ECGS was from Collaborative Research Inc. (Bedford, MA). RPMI 1640, penicillin, streptomycin, and glutamine were from Quality Biological, Inc. (Gaithersburg, MD). FBCS was from Advanced Biotechnologies, Inc.

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† Abbreviations: ELISAs, enzyme-linked immunosorbent assays; EDAC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; PEG 1500, polyethylene glycol 1500; Ig, immunoglobulin; ECGS, endothelial cell growth supplement; STM, B-cell mitogen prepared from purified cell walls of the heptoseless Re-mutant of *Salmonella typhimurium*; RAS, Ribi adjuvant system containing monophosphoryl lipid A and trehalose dimycolate; FBCS, fetal bovine calf serum; PBS, Dulbecco's phosphate-buffered saline; Triton–PBS, PBS containing 0.04% Triton X-100; BK, bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg); LBK, lysyl-bradykinin; MLBK, Methionyl-lysyl-bradykinin; −1BK, des-Arg^1-bradykinin; −9BK, des-Arg^9-bradykinin; −89BK, des-Phe^8-Arg^9-bradykinin; H, hypoxanthine; A, aminopterin; T, thymidine; and IC_{50} concentration of peptide that causes a 50% inhibition of the signal obtained with antibody in the absence of peptide.

(Silver Spring, MD). PBS was from Hazelton Research Products (Denver, PA). SP2/o cells were from the American Tissue Culture Collection (Rockville, MD). BALB/c mice were from Jackson Laboratories (Bar Harbor, ME). [^{3}H]Bradykinin (88.7 Ci/mmol) was from NEN Research Products (Boston, MA). BK was from Cambridge Research Biochemicals, Inc. (Valley Stream, NY). −1BK was from Serva Fine Biochemicals (Westbury, NY). −89BK was from Bachem Inc. (Torrance, CA). NUNC maxisorp 96-well, flat-bottom immunoplates and NUNC 96-well, flat-bottom, sterile disposable microwell plates with lids were purchased from PGC Scientific (Gaithersburg, MD). HandiSpense® was purchased from the Sandy Spring Instrument Co., Inc. (Gaithersburg, MD). Casein-Hammersten was from the United States Biochemical Corp. (Cleveland, OH).

Preparation of BK–protein conjugates. BK was coupled to KLH following the procedure of Reilly and Root [6]. Briefly, 3.5 mg of EDAC in 1 mL of water was added to 3 mL of water containing 1 mg of BK and 5 mg KLH. After incubation at room temperature for 18 hr, 0.5 mL of 0.225 M hydroxylamine hydrochloride was added and the reaction mixture incubated for an additional 5 hr at ambient temperature. Unreacted BK was separated from that conjugated to KLH by dialysis against 1 liter of PBS at 3°. PBS was replaced every 24 hr over the next 3 days.

BK was coupled to ovalbumin as follows. BK (10 mg) and 14.5 mg of ovalbumin were dissolved in 10 mL of water. EDAC (35 mg) in 1 mL of water was added to the above mixture and the pH adjusted to 7.0 with 1 N NaOH. After incubation at room temperature for 18 hr, 1 mL of 6 M hydroxylamine hydrochloride was added and the incubation continued for an additional 5 hr. Free BK was separated from that conjugated by dialysis as described above.

BK was coupled to BSA using glutaraldehyde following the procedure described by Reichlin [7] for coupling adrenocorticotropin hormone to BSA. BK (1.6 mg) and 22.4 mg of BSA were dissolved in 3 mL of 0.1 M potassium phosphate buffer, pH 7.5, in a 10-mL glass beaker. While magnetically stirring the mixture, 6 μL of a 25% aqueous solution of glutaraldehyde was added. After stirring for 18 hr at room temperature, the mixture was dialysed against PBS as described above to remove any unconjugated BK. A glycine–BSA glutaraldehyde conjugate was prepared in the same way to be used as a control in the ELISA screen described below.

Tritiated BK (1,000,000 cpm) was included with BK in each of the coupling procedures to estimate the amount of BK incorporated into each conjugate. Based on the amount of radioactivity remaining after dialysis, 13% of the BK reacted was covalently coupled with KLH, 21% with ovalbumin, and 51% with BSA.

Immunizations. A 2-month-old BALB/c mouse was immunized intraperitoneally with 0.2 mL of PBS containing 0.05 mg monophosphoryl lipid A, 0.05 mg trehalose dimycolate, 4 μL of Squalene oil, 0.2% Tween 80, and 0.11 mg BK–KLH conjugate. After 23 days, the mouse was immunized as before. Five days later the mouse was killed and spleen cells were obtained. This immunization protocol was the same as that used by Cleveland *et al.* [8] to obtain auto-anti-idiotypic antibodies reactive with the acetylcholine receptor.

A 3-month-old BALB/c mouse was given six subcutaneous injections (total volume = 0.2 mL) of immunogen, which was identical to that described above except that 0.14 mg of BK-ovalbumin conjugate replaced the BK–KLH. Booster injections of 0.2 mL of the same immunogen were given intraperitoneally every 2 weeks for a total of five boosts. Three days after the last immunization, the spleen was excised and used to prepare hybridomas.

Tissue culture media. Complete medium was prepared as follows: to 500 mL of RPMI 1640 was added 55 mL of FBCS, 5.5 mL of sodium pyruvate (100 mM), 5.5 mL of glutamine (200 mM), 5.5 mL of penicillin–streptomycin mixture (10,000 units penicillin and 10,000 μg streptomycin/mL), and 0.3 mL of 2-mercaptoethanol (100 mM).

HAT + ECGS medium was prepared by adding 11 mL of HAT stock solution (50×), which contained 10 mM hypoxanthine, 0.04 mM aminopterin, and 1.6 mM thymidine, and 15 mg ECGS to 583 mL of complete medium.

HT medium was prepared by adding 11 mL of HT stock solution (50×), which contained 10 mM hypoxanthine and 1.6 mM thymidine to 583 mL of complete medium.

HT + ECGS medium was prepared by adding 15 mg of ECGS to 594 mL of HT medium.

Fusions. SP2/o cells were grown to 85% confluency and then harvested. They were treated for 2 hr at 37° with Colcemid® (50 ng/mL) in complete medium [9]. Spleens from the immunized mice were excised under sterile conditions. Splenocytes were obtained by mashing the spleens in 1–2 mL of complete medium with a rubber plunger from a 10-mL syringe and then passing the suspension twice through a 22-gauge needle and then twice through a 25-gauge needle. The resulting cell suspension (5×10^7 cells) was mixed with the Colcemid®-treated myeloma cells (5×10^7 cells) and the volume brought to 50 mL with complete medium. Treated myeloma cells were centrifuged at 200 $g \times 10$ min and the pellet was resuspended in 20 mL of complete medium prior to mixing with the splenocytes. The mixture of splenocytes and myeloma cells was centrifuged at 2000 $g \times 10$ min and the pellet resuspended in 1 to 1.5 mL of complete medium and warmed to 37° in a waterbath. Prewarmed PEG 1500 (1 mL) was added dropwise over 1 min. The 50-mL tube was rotated gently at 37° at a 45° angle for 1.5 min. To this mixture was added 1 mL of prewarmed complete medium over 0.5 min followed by 3 mL of prewarmed complete medium over the next 0.5 min. In the next 0.5 min, an additional 17 mL of complete medium was added. The final volume was brought to 50 mL with complete medium. The tube was capped and inverted gently and left in the 37° waterbath for 5 min. The tube was centrifuged at 2000 $g \times 10$ min, and after sitting for an additional 5 min, the supernatant was carefully pipetted and discarded. The pellet was resuspended by repetitious pipetting in 120 mL of prewarmed HAT + ECGS medium [10] containing STM (5 μg/mL). Aliquots (50 μL) of this

suspension were dispensed using a pipetting device, HandiSpence®, into each well of 96-well tissue culture plates to which 50 μL of HAT + ECGS medium had already been added. After undisturbed incubation at 37° for 48 hr in a CO_2 incubator, 0.2 mL of prewarmed HT medium was added to each well. Plates were returned to the incubator and cultured until macroscopic colonies of hybridomas were detected (14–21 days). When colonies were 1–2 mm in diameter, supernatants were removed for screening.

Coating of immunoplates. Rows A, C, E, and G of plates for screening hybridoma supernatants were coated at 3° with 50 μL of a 1:3000 dilution of BK–BSA glutaraldehyde conjugate, i.e. 4.5 ng BK per well assuming all of the conjugate adsorbs to the plate. Rows B, D, F, and H were coated with 50 μL of a 1:3000 dilution of glycine–BSA glutaraldehyde conjugate. After a 1-hr incubation, the plates were inverted and the coating solutions were discarded. Plates were washed once with 100 μL of Triton–PBS at ambient temperature and then coated for 1 hr at 3° with 150 μL of 0.1% casein in 0.01 M sodium phosphate buffer, pH 7.0. After discarding the non-adsorbed casein solution, the plates were washed twice with 100 μL of Triton–PBS and then stored at 3° until needed. The casein solution had been autoclaved for 20 min to facilitate the dissolution of casein, but was cooled to ambient temperature or 3° prior to addition to the plates. Plates for the BK ELISA were coated, blocked, and stored as described above, except that all of the wells were coated with a 1:10,000 dilution of BK–BSA conjugate instead of a 1:3000 dilution of BK–BSA or glycine–BSA glutaraldehyde conjugate, i.e. 1.35 ng BK per well assuming all of the conjugate adsorbs to the plate.

Screening ELISA. Supernatants (50 μL) from wells containing hybridomas were pipetted into one of the twelve columns of the screening plate in rows A, B, C, and D or E, F, G, and H. In this way, 24 hybridoma supernatants could be assayed with one 96-well plate. After incubating for 1 hr at 3°, plates were inverted and the supernatants discarded. After washing three times with 100 μL of Triton–PBS at ambient temperature, 50 μL of a 1:350 dilution of alkaline phosphatase conjugated goat anti-mouse polyvalent immunoglobulins in Triton–PBS was added. After incubation for 1 hr at 3° plates were inverted and then washed seven times with 100 μL Triton– PBS. Fifty microliters of substrate solution, 50 mM Na_2CO_3, 1 mM $MgCl_2$, 2.7 mM *p*-nitrophenyi phosphate disodium, pH 9.8, was added and the plates were incubated for 1 hr at 37°. Reactions were terminated with the addition of 50 μL of 2 N NaOH. Absorbance at 410 nm was measured using a Dynatech MR 600 plate reader. A positive result in this assay was defined as one in which the absorbances for the wells coated with BK-conjugate were at least 0.1 greater than the wells coated with the glycine-conjugate, which in turn had an absorbance comparable to that obtained when substrate solution was incubated under assay conditions but in the absence of alkaline phosphatase. If a single colony was present in a positive well, it was transferred to a 24-well plate, grown up in HT + ECGS medium, and tested to see whether soluble BK (1 μg/10 μL) would inhibit binding of the antibody (40 μL) to the screening plate. If soluble BK inhibited binding, the hybridoma was subcloned. Two 96-well plates were seeded with an average of 3 cells/well and two plates were seeded with an average of 1 cell/well. The medium used was HT + ECGS. Cloned hybridomas were retested for inhibition by soluble BK. A few of the hybridomas from positive wells were grown up and stocked. Ascites fluid was generated in pristane-primed BALB/c mice using hybridomas which had been cloned 2–3 times. For the experiments described in this report, tissue culture supernatants containing antibody were used. If more than one colony was present in a well testing positive after the initial screen, samples of cells from each colony were transferred to separate wells of a new 96-well plate and tested for inhibition by BK after they had grown up in HT + ECGS medium. Only one of the wells testing positive after this step was subcloned, stocked, and used to generate ascites fluid.

BK ELISA. The BK ELISA was patterned after the amplification procedure described by Bjercke *et al.* [11] for nicotine and cotinine ELISA using monoclonal antibodies. A dilution of tissue culture supernatant (40 μL) containing monoclonal antibody was incubated with 10 μL of PBS or 10 μL PBS containing 1 μg of BK or six smaller amounts of BK or various amounts of BK analog. After 1 hr at 3°, the plates were inverted and then washed three times at ambient temperature with 100 μL of Triton–PBS. Goat anti-mouse polyvalent immunoglobulins (50 μL of a 1:300 dilution in Triton–PBS) were added and incubated for 1 hr at 3°. After washing as before, 50 μL of a 1:1000 dilution of alkaline phosphatase conjugated Protein A in Triton–PBS was added and incubated for 1 hr at 3°. Plates were washed seven times with 100 μL Triton–PBS and then incubated at 37° for 1 hr with 50 μL of the same substrate solution described above. Reactions were terminated with the addition of 50 μL of 2 N NaOH, and absorbance was measured as before.

Each data point was run in triplicate. The antibody dilutions were made so that the absorbance at 410 nm was between 0.5 and 1.0 when incubated in PBS. Log–dose inhibition curves were constructed by subtracting the average absorbance obtained when 1 μg of BK was included in the reaction mixtures from the averages of the absorbance values obtained for other data points and then expressing these differences as a percentage of the maximum difference, i.e. the value obtained when the absorbance in the presence 1 μg BK was subtracted from the absorbance measured when only antibody was in the incubation mixture. These percentages were plotted on the ordinate versus the log of the respective concentration of inhibitor on the abscissa. The concentrations of peptides that caused a 50% inhibition of antibody binding were determined from these curves. In all cases the absorbance obtained when 1 μg of BK was present in the incubation mixtures was the same as that obtained when kinin antibodies were omitted from the reaction mixtures, i.e. binding was completely inhibited by this amount of BK at the dilutions of kinin antibody employed. The results reported in this paper are the means ± SD of a minimum of three separate experiments with each antibody.

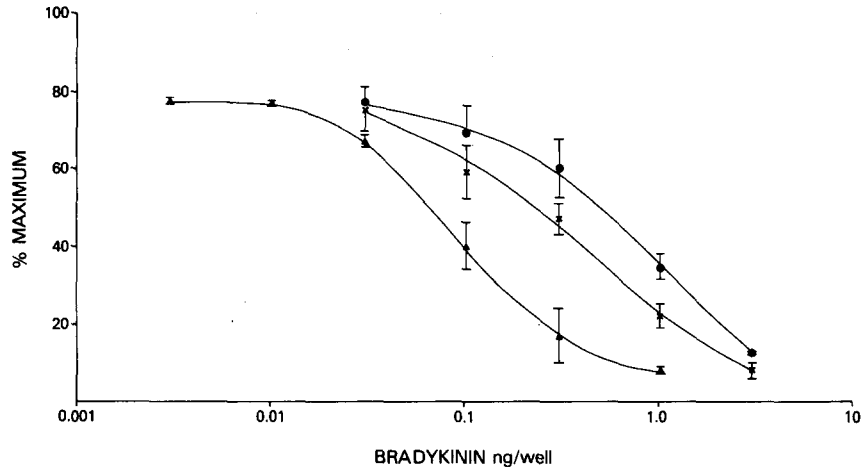

Fig. 1. BK inhibition curves of binding of OLNBK-1 (▲—▲), OLNBK-4 (×—×), and OLNBK-6 (●—●) in an ELISA. See Materials and Methods for details of the BK ELISA. Data points are the means ± SD of three separate experiments with each antibody.

Table 1. Specificities of monoclonal kinin antibodies

	IC_{50} (nM)					
		Kinin analog				
Antibody	BK	LBK	MLBK	−9BK	−1BK	−89BK
OLNBK-1	1.0 ± 0.2*	29 ± 4	24 ± 8	1.2 ± 0.2	34 ± 8	32 ± 15
	(100%)†	(3%)	(4%)	(83%)	(3%)	(3%)
OLNBK-2	2.8 ± 0.4	46 ± 8	59 ± 22	2.7 ± 0.0	91 ± 11	78 ± 14
	(100%)	(6%)	(5%)	(104%)	(3%)	(4%)
OLNBK-3	4.1 ± 1.2	97 ± 20	77 ± 12	5.3 ± 0.9	135 ± 28	22 ± 8
	(100%)	(4%)	(5%)	(77%)	(3%)	(19%)
OLNBK-4	4.2 ± 0.8	88 ± 34	71 ± 33	1.6 ± 0.5	41 ± 12	37 ± 12
	(100%)	(5%)	(6%)	(262%)	(10%)	(11%)
OLNBK-5	5.9 ± 2.7	121 ± 16	97 ± 26	1.7 ± 0.5	72 ± 28	81 ± 21
	(100%)	(5%)	(6%)	(347%)	(8%)	(7%)
OLNBK-6	7.7 ± 2.1	14 ± 6	14 ± 6	262 ± 68	47 ± 5	1960 ± 488
	(100%)	(55%)	(55%)	(3%)	(16%)	(0.4%)
OLNBK-7	8.9 ± 3.5	16 ± 5	8.7 ± 2.5	257 ± 84	55 ± 5	3869 ± 556
	(100%)	(56%)	(102%)	(3%)	(16%)	(0.2%)
OLNBK-8	22 ± 1	31 ± 11	35 ± 1	194 ± 14	97 ± 7	3912 ± 706
	(100%)	(71%)	(63%)	(11%)	(23%)	(0.6%)
OLNBK-9	39 ± 9	55 ± 5	72 ± 15	238 ± 38	118 ± 15	2608 ± 128
	(100%)	(71%)	(54%)	(16%)	(33%)	(1.5%)

* Values listed are the means ± SD of three separate experiments and are the nanomolar concentrations of analogs that inhibited by 50% (IC_{50}) the absorbance obtained when antibody was 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 BK by the IC_{50} for BK or the kinin analog in question and then multiplying by 100.

Isotyping. Dilutions of cloned BK antibodies in PBS (50 μL) were incubated for 1 hr at 3° with an immunoplate coated with a 1 : 3000 dilution of the BK–BSA glutaraldehyde conjugate and blocked with 0.1% casein as described above. After washing three times with 100 μL of Triton–PBS, rabbit anti-mouse isotype specific antibodies (50 μL) were added and incubated for 1 hr at 3°. After washing three times, alkaline phosphatase conjugated to goat anti-rabbit immunoglobulins (50 μL) was added and incubated for 1 hr at 3°. Plates were washed seven times with Triton–PBS, and 50 μL of substrate solution, described above, was added. After incubation for 30 min at 37° reactions were terminated with 50 μL of 2 N NaOH, and the absorbance at 410 nm was measured.

RESULTS

Of the 2880 wells seeded with the fusion mixture that used the spleen from the mouse immunized

with the BK–KLH conjugate, 1201 had hybridomas growing in them. Of these, 4 were positive and inhibited in the ELISA by soluble BK. However, only 1 (OLNBK-5, Table 1) of the 4 positives was stable and survived subcloning. Apparently the other 3 died or stopped secreting antibody prior to their being screened, since none of the hybridomas transferred to the 24-well plate and subcloned tested positive for BK antibody.

Seven hundred and ninety of the 2880 wells seeded with the fusion mixture that used the spleen from the mouse immunized with the BK–ovalbumin conjugate had hybridoma growth. Eight of these were found to be producing kinin antibody. All were stable and survived subcloning.

The specificities of the monoclonal antibodies were evaluated with BK and five analogs. The results of these evaluations are recorded in Table 1. The antibodies are listed in descending order of affinity for BK, based on the IC_{50} obtained with this peptide. The antibodies could be separated into three groups as determined by their relative specificities. The first group, OLNBK-1, -2, and -3, which also had the highest affinities for BK, displayed about equal reactivities for BK and −9BK, but little reactivities for the kinin fragments −1BK or −89BK, or for LBK and MLBK. The second group, OLNBK-4 and -5, was similar to the first except reactivities with −9BK were about 2.5 to 3.5 greater than for BK. The third group, OLNBK-6, -7, -8, and -9, which had the lowest affinities for BK, IC_{50} for BK ranging from about 8 to 39 nM, showed little reactivities with −1BK, −9BK, and −89BK, but 50–100% cross-reactivities with LBK and MLBK.

The log–dose response curves obtained with BK for the highest affinity antibody in groups 1, 2, and 3, OLNBK-1, OLNBK-4, and OLNBK-6, respectively, are shown in Fig. 1. The useful ranges for BK detection (ng/well, 50 μL assay volume) were 0.01 to 0.5, 0.03 to 3, and 0.1 to 3 for OLNBK-1, OLNBK-4, and OLNBK-6, respectively.

All of the antibodies were of the IgG_{1k} isotype except for OLNBK-5, which was an IgG_{2ak}. The hybridomas were all able to grow after cryopreservation, and when injected into pristane-primed mice the ascite fluids produced contained kinin antibodies.

DISCUSSION

There have been two previous papers [12, 13] on the production of monoclonal antibodies reactive with kinins. In the first paper, the affinities and specificities of the antibodies were primarily assessed using a radioimmunoassay procedure. In the present report, ELISAs were used to characterize the antibodies and, therefore, a direct comparison between our results and those of Bedi and Back [12] would not be appropriate. However, we have evaluated their antibodies using our ELISA procedures. Concentrations of BK that caused a 50% inhibition of the signal obtained in the absence of added BK were: 11.7 ± 4.2, 28.2 ± 6.7, 265 ± 106, and 1025 ± 578 nM for BK-D6A5, BK-B6C9, BK-A3D9, and BK-D2C7 respectively. Preliminary assessment of the specificities of these antibodies using our assay conditions yielded results similar to those previously published [12]. BK-D6A5 and BK-B6C9 most clearly resemble our third group of antibodies, OLNBK 6–9, with respect to affinity and specificity. None of the antibodies we obtained had low affinities or cross-reactivities with −1BK of 100% or greater like BK-A3D9 and BK-D2C7. Clearly, the reason that we do not obtain antibodies with these properties is not due to the inability of our ELISA to detect such antibodies, but may simply be due to the fact that hybridomas producing such antibodies were never formed in our fusions or, if formed, did not survive.

Bedi and Back [12] used the same coupling agent for preparing the BK-immunogen and BK-conjugate used for ELISA screening. Since Briand *et al.* [14] have reported the difficulties that can arise as a result of this choice, in the present study a different coupling agent (glutaraldehyde) was used in the preparation of the BK-conjugate for screening than was used for the preparation of the BK-immunogen (EDAC).

The usual way for screening for monoclonal antibodies reactive with a peptide is to coat a protein conjugate of the peptide on the screening plate and then, after blocking any remaining sites on the plate with a protein solution, to incubate the hybridoma supernatant in the presence or absence of soluble peptide. Those supernatants whose signal is reduced by the inclusion of the soluble peptide are selected for further evaluation. This was the approach we initially employed. However, most of the hybridomas selected, when subjected to further analyses, were not producing antibodies reactive with BK. This may have been due to a "non-specific" interference by the soluble BK, a basic peptide, of the binding of the mouse antibody, an acidic protein, to a positively charged region of the BK–BSA conjugate used to coat the plate. In addition, there were a number of hybridoma supernatants that yielded a high signal when incubated with the plate coated with the BK-conjugate, but were not inhibited at all by the presence of soluble BK. These could have been hybridomas producing kinin antibodies in such high concentrations that the 1 μg of soluble BK added was not sufficient to inhibit antibody binding to the plate. Dilutions of these hybridoma supernatants were required to evaluate this possibility, but this could only be done after feeding the wells containing such hybridomas and re-evaluating them 2 days later.

To overcome these limitations of the more conventional approach to screen for anti-peptide antibodies, the procedure described in this report was developed. The screen is based on the specificity of binding. By using the same coupling agent to prepare BK and glycine conjugates used to coat the screening plates, antibodies, which are reactive with the bond formed by the coupling agent, yield comparable signals when incubated with wells coated with either of these conjugates. Antibodies reactive with BSA or with the casein blocking agent yield a similar result. The amount of BK-conjugate to coat on the plate was determined by coating various amounts of the conjugate on the plate and using the plate to see which dilution of coating agent gave a good signal

when a high-affinity polyclonal rabbit BK antibody was reacted with the plate.

Every hybridoma supernatant tested, whose absorbance for wells coated with the BK-conjugate were at least 0.1 greater than the wells coated with the glycine-conjugate, which in turn had an absorbance comparable to the blank, contained antibody reactive with kinins. None of the hybridoma supernatants was found to contain kinin reactive antibodies when the signals on the wells coated with the BK-conjugate were greater than those obtained with wells coated with the glycine conjugate, but the signals on the glycine-conjugate coated wells were greater than the blank.

Since the screening assay developed does not depend on the inhibition of antibody binding to the plate by soluble peptide, like more conventional screening assays, hybridomas secreting large amounts of antibody are determined in the first screen as containing kinin reactive antibody. No time is wasted in following-up on supernatants whose strong signal is weakly inhibited by soluble BK due to "non-specific" ionic effects. Investigators embarking on studies to prepare monoclonal antibodies reactive with other peptides or proteins should consider the approach taken in the present study when deciding on how to screen for the antibodies of interest.

Except for OLNBK-5, an IgG_{2ak}, all of the antibodies obtained were subclass IgG_{1k} as were those reported by Bedi and Back [12] and Chao *et al.* [13]. Chao *et al.* [13] provided no data on the affinity of their two monoclonal antibodies for BK but the apparent cross-reactivities of their antibodies with MLBK and LBK lead us to believe that they would be very similar to our third group of antibodies, OLNBK 6–9. Although none of our antibodies were tested for their abilities to react with kininogen, it is likely that OLNBK 6–9 would react with this kinin precursor given their apparent similarities to monoclonal BK antibodies previously described [12, 13]. It is less likely that groups 1, OLNBK 1–3, or 2, OLNBK 4–5, will react with kininogen, given these groups demonstrate relatively little cross-reactivities with MLBK and LBK.

The useful range for ELISA detection of BK using OLNBK-6 was 0.1 to 3 ng in a final assay volume of 50 μL, i.e. a concentration range of 2–60 ng/mL. OLNBK-6 was the highest affinity monoclonal antibody that reacts well with BK, LBK and MLBK, kinins active on a B2 BK receptor, but not with −9BK, the kinin active on B1 BK receptors. Although this range of detection is a little higher than that reported by Geiger and Miska [15] for their ELISA using a polyclonal rabbit BK antibody, 0.5 to 500 ng/mL, it is an order of magnitude better than that reported by Bedi and Back [12], who also used a monoclonal BK antibody in their ELISA, 50–1500 ng/mL.

The ELISAs presently are not sensitive enough to be used to detect BK in normal blood and urine, unless these fluids are processed in some way so as to concentrate them before analysis. However, the ELISAs could be used to monitor the release of kinin from kininogen in kininogenase assays and may be adequate for the measurement of kinins in biological fluids in disease states associated with elevated kinins, e.g. hereditary angioneurotic edema, septic shock, and carcinoid syndrome or in experimental animal models of inflammation [1].

The monoclonal antibodies obtained, because of their relatively high affinities for BK, are likely to be able to block the actions of BK by competing with receptors for any available kinin. It may even be possible to determine whether a B1 or a B2 BK receptor is invoked. If OLNBK-6 blocks, it is likely that a B2 BK receptor is invoked, because this antibody binds kinins with a specificity most like a B2 BK receptor. If OLNBK-4 blocks, but OLNBK-6 does not, then a B1 BK receptor is probably involved, since this antibody binds kinins with a specificity most like a B1 BK receptor. Following the same line of reasoning, OLNBK-6, -7, -8 and -9 would be more likely to elicit anti-idiotypic antibodies that cross-reacted with B2 BK receptors and OLNBK-4 and -5 would be more likely to elicit anti-idiotypic antibodies that cross-reacted with B1 BK receptors. Experiments are being initiated to test the validity of these hypotheses.

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REFERENCES

1. Proud D and Kaplan AP, Kinin formation: Mechanisms and role in inflammatory disorders. *Annu Rev Immunol* **6**: 49–83, 1988.
2. Sharma JN, Interrelationship between the kallikrein–kinin system and hypertension: A review. *Gen Pharmacol* **19**: 177–187, 1988.
3. Regoli D and Barabe J, Pharmacology of bradykinin and related kinins. *Pharmacol Rev* **32**: 1–46, 1980.
4. Vavrek RJ and Stewart JM, Competitive antagonists of bradykinin. *Peptides* **6**: 161–164, 1985.
5. Köhler G and Milstein C, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495–497, 1975.
6. Reilly TM and Root RT, Production of idiotypic and anti-idiotypic antibodies by BALB/c mice in response to immunizations with glucagon, vasopressin, or insulin: Supporting evidence for the network concept. *J Immunol* **137**: 597–602, 1986.
7. Reichlin M, Use of glutaraldehyde as a coupling agent for proteins and peptides. In: *Methods in Enzymology* (Eds. Van Vunakis H and Langone JJ), pp. 159–165. Academic Press, New York, 1980.
8. Cleveland WL, Wassermann NH, Sarangarajan R, Penn AS and Erlanger BF, Monoclonal antibodies to the acetycholine receptor by a normally functioning auto-anti-idiotypic mechanism. *Nature* **305**: 56–57, 1983.

9. Miyahara M, Nakamura H and Hamaguchi T, Colcemid treatment of myeloma prior to cell fusion increases the yield of hybridomas between myeloma and splenocyte. *Biochem Biophys Res Commun* **124**: 903–908, 1984.
10. Pintus C, Ransom JH and Evans CH, Endothelial cell growth supplement: A cell cloning factor that promotes the growth of monoclonal antibody producing hybridoma cells. *J Immunol Methods* **61**: 195–200, 1983.
11. Bjercke RJ, Cook G, Rychlik N, Gjika HB, Van Vunakis H and Langone JJ, Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. *J Immunol Methods* **90**: 203–213, 1986.
12. Bedi GS and Back N, Monoclonal antibodies to bradykinin inhibit smooth muscle contractile action of bradykinin. *Biochim Biophys Acta* **842**: 90–99, 1985.
13. Chao J, Swain C, Chao S, Xiong W and Chao L, Tissue distribution and kininogen gene expression after acute-phase inflammation. *Biochim Biophys Acta* **964**: 329–339, 1988.
14. Briand JP, Muller S and Van Regenmortel MHV, Synthetic peptides as antigens: Pitfalls of conjugation methods. *J Immunol Methods* **78**: 59–69, 1985.
15. Geiger R and Miska W, Determination of bradykinin by enzyme immunoassay. *Adv Exp Med Biol* **198** (Pt. B): 531–536, 1986.