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Short Communication

Preparation and Characterization of Specific and High-Affinity Monoclonal Antibodies Against Morphine

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ABSTRACT

A C6-hemisuccinate derivative of morphine was prepared and conjugated to bovine serum albumin. High titer antibody producing spleen cells were removed and fused with myeloma cells of Sp2/0 origin. A C3-hemisuccinate derivative of morphine was prepared and conjugated to enzyme penicillinase used as a tracer molecule. A novel enzyme-linked immunoadsorbent assay was developed using this conjugate to screen and characterize the monoclonal antibody produced in these experiments. After two successive limiting dilutions, antibodies produced by 5 clones with good affinities ranging from 10^8 to 10^{12} M^{-1} and less cross-reaction (least for codeine and other structurally related molecules) were selected. These clones were found to be of IgG class with κ light chain. Subclass determination showed that two of the clones produced IgG2b and three of them produced IgG1 type of antibody. Affinity purifications were performed for the selected clone (MOR-I). Purified antibody was coated onto the wells of microtiter plate. The standard curve was constructed with a sensitivity of 100 pg/mL covering up to 10 ng/mL in buffer and urine. The slope of the standard curve for selected clone in buffer and urine was calculated to be -0.7 and -0.64, respectively.

INTRODUCTION

MORPHINE (7,8-Didehydro-4,5-epoxy-3,6-dihydroxy-*n*-methyl morphinan) is obtained as a milky extract of plant papaver somniferum. This compound is a known μ receptor agonist and is therefore used as an efficient analgesic agent.[1] Unfortunately, this compound is mostly used as an abused drug. Iran is situated among those countries producing a large amount of opioids. The product is mostly transited through Iran to western European countries. The situation is so devastating that out of 11 tons of morphine confiscated around the world during the year 1996, 10 tons were seized in Iran alone.[2] Therefore, it is essential to develop simple, sensitive, specific and high throughput assays to measure morphine in biological samples. Numbers of chromatographic assays are reported for morphine and its metabolites, such as TLC,[3] GC,[4] HPLC,[5,6] GC/MS,[7] and HPLC/MS.[8] However, these methods are time-consuming and difficult to perform. Immunochemical methods are more rapid and easy to perform. Radioimmunoassay (RIA), hemagglutination inhibition, and enzyme-linked immunoadsorbent assay (ELISA) are reported for morphine detection in biological samples.[9–14] In these assays antibody plays a crucial rule because the specificity largely depends upon the antibody quality.[15] Reports on polyclonal and monoclonal antibodies (MAbs) against morphine are available.[16–20] In these, specificity and affinity are of prime importance. In this study, we have produced a specific and high-affinity antibody against morphine and characterized them with a novel ELISA using penicillinase as label that was previously reported for a number of hapten molecules.[21–23]

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TABLE 1. CLONES SELECTED FOR FURTHER EXPERIMENTS AFTER FUSION AND FIRST LIMITING DILUTION

Clones selected after fusion				*Clones selected after first limiting dilution*				
		OD at 450 nm				*OD at 450 nm*		
Clones	*OD at 600 nm*	*M-TG*	*M-OVA*	*Clones*	*OD at 600 nm*	*M-TG*	*M-OVA*	*Affinities*
Control (Sp2/0)	1.4	0.14	0.15	Control (Sp2/0)	1.4	0.14	0.15	b
N_1B_7	0.84	1.08	1.10	$B_{11}F_6$	0.78	0.96	0.98	2.85×10^{12}
N_2B_{11}	0.72	1.25	1.24	$B_{11}E_7$	0.71	1.12	1.11	2×10^{9}
N_2B_{11}	0.78	1.12	1.12	E_7D_8	0.8	0.95	0.98	2×10^{10}
N_2B_5	0.78	1.12	1.10	$G_{10}F_{11}$	0.81	0.93	0.91	3×10^{12}
N_3A_6	0.79	1.07	1.08	$B_{11}F_7$[a]	0.72	1.21	1.20	1.25×10^{11}
N_3C_4	0.83	1.08	1.08	E_7D_7	0.65	1.31	1.30	2.3×10^{11}
N_3E_2	0.84	1.09	1.08	$G_{10}F_{10}$[a]	0.8	0.95	0.96	1×10^{11}
N_3F_8	0.82	1.09	1.09	$B_{11}D_3$	0.78	1.10	1.10	4.3×10^{11}
N_3G_{10}	0.83	0.85	0.88	E_7C_4	0.8	0.86	0.88	2.8×10^{11}
N_3E_7	0.9	0.7	0.7	$G_{10}D_2$[a]	0.78	1.0	1.01	7.1×10^{12}
N_3H_{11}	0.76	1.12	1.13	E_7E6	079	0.95	0.96	1.2×10^{12}
N_3H_9	0.78	1.10	1.09	$G_{10}D_8$[a]	0.67	1.21	1.2	8.3×10^{11}
				$G_{10}F_8$[a]	0.65	1.31	1.30	3×10^{12}
				$B_{11}F_9$[a]	0.68	1.29	1.30	1.16×10^{12}
				E_7D_4[a]	0.81	0.71	0.73	2.8×10^{12}

OD, optical density; M-OVA, morphine conjugated ovalbumin; and M-TG = morphine conjugated thyroglobulin.
[a]Selected clones for second limiting dilution.
[b]OD at 450 nm for TG or OVA is between 0.13 to 0.36.

MATERIALS AND METHODS

RPMI 1640, polyethyleneglycol (PEG), fetal calf serum (FCS), penicillinase (β-lactamase, EC.3.5.2.6), penicillin V, streptomycin, hypoxanthine–aminopterin–thymidine (HAT), HT, morphine-like compounds, bovine serum albumin (BSA), anti-mouse IgG-horseradish peroxidase (IgG-HRP), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), tetramethylbenzidine (TMB), and pristane were all obtained from Sigma Chemical Company (St. Louis, MO). ELISA plates (96 wells) and other plastic wares were obtained from Nunc (Denmark). Myeloma cell line of Sp2/0 origin was obtained from Pasteur Institute (I.R. Iran). Two-month-old BALB/c mice were obtained from Razi Research Institute (I.R. Iran). All other reagents used in this study were of analytical grade and obtained from standard sources.

Immunization

Immunogen was prepared following a previously explained procedure.[24] Ten female BALB/c mice were immunized intraperitoneally primarily with 75 μg of immunogen in CFA and later with the same amount of immunogen prepared in IFA for booster injections, each 15 days. Serum samples were prepared each month and high-titer antibody-producing animals were sacrificed for hybridoma formation after 9 months of continuous immunization.

Antibody detection

To detect antibody in serum, cell culture supernatant or ascitic fluid, a C3-hemisuccinate derivative of morphine, was prepared[17] and conjugated to the enzyme penicillinase, following carbodiimide procedure.[24] For polyclonal antibody detection, purified anti-IgG of normal mouse prepared in rabbits was coated onto the wells of microtiter plates at 37°C over night, washed, and blocked with a 0.5% solution of gelatin supplemented phosphate-buffered saline (PBS) (10 mM, pH 7.2) for 30 min at 37°C, washed and added with dilutions of polyclonal antimorphine antibodies (serum). In each experiment, normal mouse serum (NMS) with a dilution of 1:300 was used for non-

TABLE 2. STANDARD CURVES OF FIVE FINALLY SELECTED CLONES AFTER TWICE LIMITING DILUTION TERMS OF PERCENT BINDING FOR EACH DOSE OF MORPHINE

	Percent binding				
	Clones				
Doses (pg/mL)	*MOR-I*	*MOR-II*	*MOR-III*	*MOR-IV*	*MOR-V*
0	100	100	100	100	100
100	66	59	90	80	80
1000	33	17	80	60	66
10,000	3	3	60	20	50

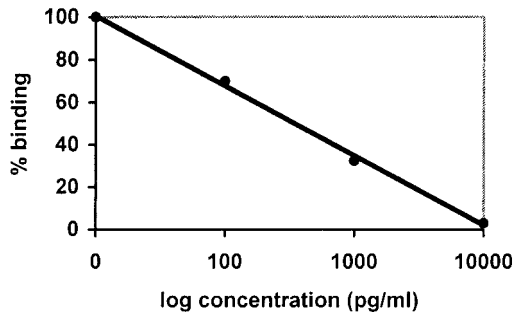

FIG. 1. Standard curve of morphine in buffer for purified MAb (MOR-I).

specific binding (NSB). Index content of the wells were incubated at 37°C for 2 h, washed, added to morphine-penicillinase conjugate, and incubated at 37°C for 1 h. At the end of incubation time, wells were washed and added with 100 μL of 0.2 M/L freshly prepared substrate solution penicillin V, as explained previously.[23] Plate was incubated at 37°C for 1 h and added with 150 μL of starch-iodine solution prepared as reported previously.[25] The reaction mixture was incubated at room temperature for 10 min and the color development was measured at 600 nm. In another set of experiments, C6-morphine hemisuccinate was conjugated to either thyroglobulin or ovalbumin, sufficient amount of the product was coated onto the wells, incubated, washed, blocked, and added with serum samples. After incubation at 37°C for 2 h, plates were washed and added with proper dilution of anti-mouse IgG-HRP, incubated, and washed. Wells were treated with 100 μL of substrate TMB and incubated for 10 min. The enzymatic reaction was terminated using 50 μL of a 2 N sulfuric acid solution and the color development was measured at 450 nm.

Hybridoma formation

High-titer antibody-producing animals selected in the above method were sacrificed; spleen cells were removed and fused with pretreated Sp2/0 myeloma cells in a 40% solution of PEG. Hybridomas were cultured in a 20% FCS containing medium supplemented with HAT for 3 weeks. Positive clones were selected following the procedure explained below and subcultured in a 20% FCS containing medium for another 2 weeks. Supernatant of the wells containing a monoclonal cell growth was characterized for titer, specificity, and affinity. Proper clones were selected, diluted for a second time, subcultured, and characterized for specificity, affinity, titer, class, and subclass identification. Selected antibody-producing clones (MOR-I and MOR-II) were cultured in 50-mL flasks or alternatively cells were injected intraperitoneally to pristane pretreated mice. Supernatant or ascitic fluid were collected and purified using ammonium sulfate precipitation and protein A coupled agarose column. Purified immunoglobulin was used to construct standard curve in buffer and urine.

MAb detection and characterization

To screen antibody in supernatant of cell culture medium, purified anti-mouse normal IgG antibodies were coated onto the wells of microtiter plates, as explained previously, added with supernatant, incubated for 2 h, and washed. The rest was performed, as explained previously. The supernatant was also checked with HRP label as a confirmation test, the same as reported for polyclonal antibody. For antibody detection in ascitic fluid, the clear supernatant was coated, blocked, washed, and dilutions of morphine-penicillinase were added. The rest was performed as explained previously.

Standard curve was constructed in buffer and urine using MAb-purified supernatant. For this purpose, the purified MAb was coated (7 μg/well), blocked, and washed. Different doses of morphine standard preparation in PBS (10 mM, pH 7.2, containing 0.1% of gelatin and 0.1% sodium azide) or urine from 100 pg/mL up to 10,000 pg/mL was added and incubated for 1.5 h, added with C3 morphine-penicillinase, and incubated for 1 h. The rest of the experiments followed as explained previously. For cross-reactivity study, morphine-like compounds (100 pg/mL up to 10 ng/mL) were added in place of morphine.

Alternatively, using HRP-IgG as label, the standard graph was also prepared in these assays. Supernatant of cell culture medium after first- and second-limiting dilution were reacted with morphine- or morphine-like compounds in another container and transferred into the morphine-thyroglobulin or morphine-ovalbumin precoated microtiter wells, washed, and added to HRP-IgG, and incubated for 1 h. The rest was performed as mentioned previously. Class and subclass determination was performed using Roche isostrip test as advised by the manufacturer.

RESULTS

Table 1 shows the results of antibody-producing clones after fusion and first-limiting dilutions. Five clones with highest titer, affinity, and cell growth were selected, which are denoted by asterisks. Note that optical densities (O.D.), when penicillinase was used as label, decreased, while it increased when peroxidase was used as trace. The result of percent binding in dose–response curve prepared in buffer is shown in Table 2. Antibodies produced out of all five selected clones could detect morphine from 100 pg/mL and the standard curve covered up to 10 ng/mL. In case of antibody obtained from one of the clones (MOR-I) that was purified and used to prepare a standard curve in buffer and urine, sensitivity from 100 to 10,000

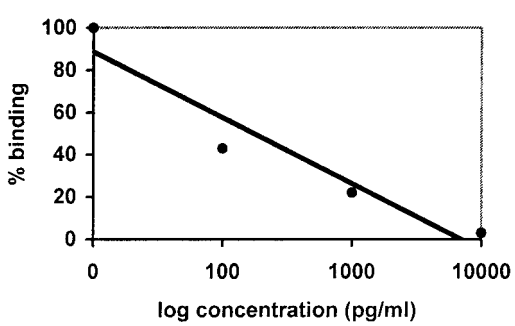

FIG. 2. Standard curve of morphine in urine for purified MAb (MOR-I).

TABLE 3. CHARATERIZATION OF SELECTED CLONES WITH RESPECT TO THEIR SPECIFICITIES, AFFINITES, AND CLASS AND SUBCLASS DETERMINATION

Monoclones	*MOR-I*[a]	*MOR-II*	*MOR-III*	*MOR-IV*	*MOR-V*
Isotyping	G1, κ	G2b κ	G1, κ	G1, κ	G2b, κ
Affinities	2.8×10^{12}	3×10^{12}	2.2×10^{8}	2.2×10^{9}	8.3×10^{11}
Inhibitors			*Percent binding*		
Morphine	100	100	100	100	100
Codeine	0.001	30	0.1	6	16
Heroin	100	173	10	52	19
Naloxan	2	24	0.1	52	10
Mehtadone	28	24	0.1	100	16
M-3-G	0.001	0	0.1	0.1	0
Apomorphine	75	79	10	0	24
Theophylline	0	0	0	100	11
Ethylmorphine	1	3	10	0	19
Cocaine	12	0	0	22	0
Caffeine	0	0	0	130	13
Thebaine	2	79	10	121	14
Salicylic acid	0	0	10	0	10
Ephedrine	0	0	0	100	29

[a]Selected clone for purification and further investigation.

pg/mL of morphine was observed using penicillinase as a label with a slope of -0.7 and -0.64, respectively (Figs. 1 and 2). Table 3 summarizes the characterization of selected clones in term of specificity (i.e., cross-reaction with 14 structurally related molecules) some of them showing much less cross-reaction with produced antibodies, such as MOR-I exhibiting no cross-reaction with codeine, morphine-3-glucuronide (M-3-G), theophylline, ethylmorphine, caffeine, salicyclic acid, and ephedrine. In this table the results of class and subclass determination is also reported, demonstrating that the antibodies are of G class, G1 and G2b subclass and all contained κ light chain. These are of high-affinity type antibodies ranging from 10^8 to 10^{12} M^{-1}.

DISCUSSION

Immunochemical techniques are used for rapid determination of morphine. This is essential because widespread drug abuse needs to be controlled. Two important components of immunoassays are antibody and labeled antigen or antibody. MAbs are highly useful in the development of immunoassay for hapten drug molecules. To prepare specific and sensitive immunoassays for morphine, polyclonal antibodies are reported with various specificities.[9–14] One important strategy is to chemically modify the hapten molecule to obtain better specificities. This strategy has been an effective measure in other cases such as testosterone[23] and progesterone.[26] The modulation of the antibody specificity of anti-hapten antibodies was reviewed by Chappey et al.[27] However, cellular modifications are considered more effective and MAbs for hapten with good specificities are reported.[28–30] MAbs against morphine are also reported; these are of various specificities.[18–20] In these type of strategies, sometimes chemical modifications of hapten molecule, as well as modification of antibody-producing cells, are taken into consideration together. Because of these types of modifications, Usagawa et al.[28] could obtain antibodies of high specificities even for codeine, as reported to exhibit negligible cross-reaction. We have prepared MAbs against a common derivative of morphine (i.e., C6-morphine hemisuccinate-BSA). One clone out of five clones prepared in this study provided highly specific antibodies against morphine (MOR-I having no cross-reaction for codeine and morphine-3-glucuronid). The affinities calculated for this antibody by Scatchard analysis (2.8×10^{12} M^{-1}) showed that it was of very high affinity. Linearization of standard curve prepared in PBS and urine indicated a slope of -0.7 and -0.64, respectively, for MOR-I. The standard curve constructed using ammonium sulfate precipitation and protein A purified antibody in buffer as well as in urine used as a carrier for standard doses showed a sensitivity from 100 pg/mL, covering up to almost 10,000 pg/mL of standard concentration of morphine. The immunostrip test for class and subclass determination indicated that most of the clones were of IgG1 and IgG2b subclass with κ light chain. In our experiments, a novel ELISA method using penicillinase as label enzyme was used. This procedure proved to be simple and sensitive and because the color development was well in the range of visible light (600 nm), a naked eye detection for antibody containing wells were possible such that a noncontaining antibody wells, weakly containing antibody wells, and strongly containing antibody wells could easily be recognized. Using this method, it was also possible to detect concentration of morphine in PBS or urine visually. This procedure was found to be more practical than HRP-IgG for rapid screening of the antibody. To the best of our knowledge, this is the first report on the use of penicillinase as a label enzyme for supernatant screening of antibody-producing cells.

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