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Production and Characterization of Specific Antibodies to Bombesin

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=ABSTRACT=

In order to produce a specific bombesin antiserum for very sensitive radioimmunoassay, synthetic [lys^3]-bombesin conjugated to bovine serum albumin was subcutaneously injected into guinea pigs. The conjugation was performed using either carbodiimide or glutaraldehyde as a coupling agent. The antisera were characterized by analysis of Scatchard and Sips plots. The antiserum LBE 2G/2 raised by repeat injection of the immunogen conjugated with carbodiimide showed the titer of 1 : 188,000, very low cross-reactivity to bombesin-like peptides except bombesin, with high affinity constant (1.64×10^{11} M^{-1}) and high heterogeneity index (0.91). The antiserum LBG 1G/2 produced by repeat injection of the immunogen conjugated with glutaraldehyde possessed the titer of 1 : 43,000, high cross-reactivity to some bombesin-like peptides, high affinity constant (1.19×10^{11} M^{-1}) and high heterogeneity index (0.79). These results indicate that the antiserum LBE 2G/2 is specific only to bombesin and that the antiserum LBG 1G/2 binds to some bombesin-like peptides such as alytesin, gastrin releasing peptide and neuromedin C. The antiserum LBE 2G/2 is sufficient for the very sensitive radioimmunoassay of bombesin.

Key Words; Bombesin antibody, Immunization technique, Radioimmunoassay

INTRODUCTION

Bombesin is a peptide originally isolated from the skin of European frogs (Erspamer et al, 1970). At the present time, a number of peptides belonging to the bombesin family (bombesin-like peptide) have been isolated from amphibian and mammalian tissues (Bevin & Zasloff, 1990; Lebacq-Verheyden et al, 1990; Nagalla et al, 1992). It has been known that bombesin exerts numerous biological activities including gastrointestinal (Bertaccini et al, 1973; Konturek et al, 1976; Scarpignato & Bertaccini, 1981), neurological (Koninck & Henry, 1989; Flynn, 1992) and immunological (Moore, 1984; Ruff et al, 1985) functions. Bombesin is also considered to be a growth factor particularly in small cell carcinoma of the lung (Weynants et al, 1990). Therefore, the development of a sensitive determination method for bombesin in the biological fluids is essential for the study of its biological activities.

Radioimmunoassay can provide a highly sensitive and reliable measurement of the peptide concentration in the various biological fluids. In order to increase the sensitivity and specificity of the radioimmunoassay, the production of a suitable antibody along with preparation of a tracer is very important (Hurn & Landon, 1971). It is known that an antiserum raised against a polypeptide recognizes only tri-

to hexa-peptide sequence of the peptide (Rehfeld & Morley, 1983). Therefore, it is difficult to develop the radioimmunoassay for a peptide that has naturally occurring heterogenous forms because of cross-reactivity of the antibody.

All of bombesin-like peptides have been known to possess biological and immunological activities and amino acid sequences, particularly at the carboxyl-terminal region, which are very similar to bombesin. Although a number of antibodies against bombesin have been reported (Erspamer et al, 1979; Walsh et al, 1979; Ghatei et al, 1982; Major et al, 1983; Reeve et al, 1983), most of them appear to be directed to the carboxyl-terminal region of bombesin. Therefore, the antibodies seem not to be appropriate for use in the very sensitive radioimmunoassay of bombesin because of its high cross-reactivity.

In the present communication, we report a specific bombesin antiserum possessing very low cross-reactivity to other bombesin-like peptides except bombesin. Therefore, the antiserum can be used for the very sensitive radioimmunoassay of bombesin.

METHODS

Production of bombesin antibodies

In order to produce bombesin antibodies, synthetic [lys^3]-bombesin (Peninsula, USA) conjugated to bovine serum albumin (Fr. V; Sigma, USA) was used as an immunogen. In one group of experiments, the conjugation was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimmide (Sigma, USA) as a coupling agent, according to the method of Boehm et al (1974). Briefly, [lys^3]-bombesin (2 mg) and bovine serum albumin (20 mg) were dissolved in 3.8 ml of deionized water and then mixed with carbodiimide (200 mg) to a final volume of 4 ml. The mixture was continuously stirred for 20 h at room temperature. In another group of experiments, the conjugation was carried out using glutaraldehyde (Merck, USA) according to a modified procedure of Reichlin (1980). In brief, [lys^3]-bombesin (2 mg) and bovine serum albumin (20 mg) were dissolved in 4 ml of 25 mM NaH_2PO_4 buffer (pH 8.0) and then mixed with 30 μl of 10% glutaraldehyde. The mixture was stirred for 4 h at room temperature. Both conjugates were finally dialyzed against 5 liters of 10 mM NaH_2PO_4 buffer (pH 7.4) containing 150 mM NaCl for 48 h at 4°C. The dialyzed conjugated immunogens were emulsified in an equal volume of complete Freund's adjuvant (Sigma, USA). A 200 μl aliquot of the emulsion, containing 50 μg of [lys^3]-bombesin, was subcutaneously injected into the back of 3 guinea pigs, respectively. Two weeks after the first injection, the second immunization was performed. Thereafter, the immunization was repeated every 4 weeks a period of 7~8 months. Blood was sampled by heart puncture 2 weeks after each immunization. The titer of bombesin antibody in the serum was determined by measuring the binding of 1.5 fmole (approximately 5,000 cpm) ^{125}I-[tyr^4]-bombesin to the serially diluted antiserum and expressed as the dilution of the antiserum at which 50% of the tracer was bound.

Characterization of bombesin antibodies

Specificity: The specificities of the bombesin antisera were assessed by the ability of some relevant bombesin-like peptides and gastrointestinal peptides to compete with ^{125}I-[tyr^4]-bombesin for binding sites of the antibodies (Chang & Chey, 1983). The extent of cross-reactivity was expressed as the half-saturation concentration ($C_{0.5}$).

Affinity and heterogeneity: Since an antibody is occasionally heterogenous with respect to affinity for the antigen (Karush, 1962), the characteristic parameters reflecting the heterogeneity of the antibody population can also be obtained by thermodynamic measurement (Chang & Chey, 1980). The affinity constants, total binding sites and heterogeneity indices of the bombesin anti-bo-

dies were estimated according to the previously described methods (Chang & Chey, 1980; Park et al, 1989a). Briefly, the effective affinity constant (K_{eff}) and total number of bombesin binding sites (N) were calculated from the initial slope and the extrapolated intercept at the abscissa of the Scatchard plot (Scatchard, 1940). The Sips equation (Sips, 1948) was used to calculate the heterogeneity index (α) and average affinity constant (K_0).

Radioimmunoassay of bombesin

Radioimmunoassay of bombesin was performed according to the method described previously by Park et al (1989b). For preparation of a tracer, [tyr^4]-bombesin (Peninsula, USA) was iodinated using Na^{125}I (Amersham, England) and lactoperoxidase (Bachem, USA). ^{125}I-[tyr^4]-Bombesin was purified by injection into a reversed phase C_{18} column (ODS-120T, 4.6×250 mm) attached to a high performance liquid chromatograph (LKB, Sweden). The specific radioactivity of ^{125}I-[tyr^4]-bombesin used in the present radioimmunoassay was 2,400 μCi nmole^{-1} as determined by the self-displacement method (Stadil & Rehfeld, 1972).

RESULTS

Production of bombesin antibodies

All six guinea pigs, immunized with [lys^3]-bombesin conjugated to bovine serum albumin by using either carbodiimide (carbodiimide group) or glutaraldehyde (glutaraldehyde group), produced detectable antibodies after the second or third immunization. The antibody titer gradually rose depending upon the number of immunization and reached to the maximal level after the eighth or ninth injection. The antiserum LBE 2G/2 of the carbodiimide group and LBG 1G/2 of the glutaraldehyde group showed the highest titer of 1 : 188,000 and 1 : 43,000, respectively after

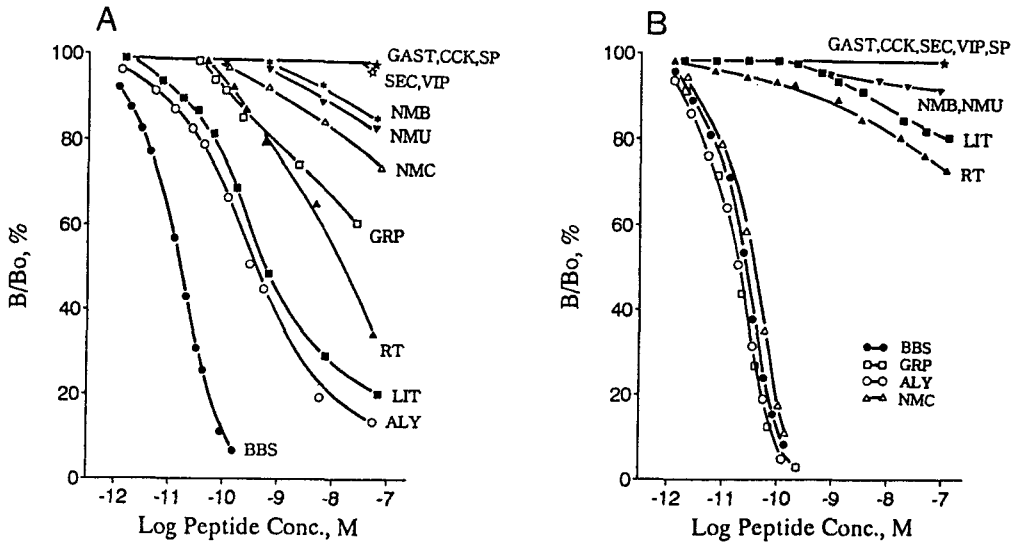

Fig. 1. Cross-reactivity of bombesin antisera, LBE 2G/2 (A), LBG 1G/2 (B). Extent of the cross-reactivity was expressed as tracer binding ratio at various peptide concentrations comparing with it that of zero concentration (Bo). Abbreviations: BBS; bombesin, ALY; alytesin, LIT; litorin, RT; ranatensin, GRP; gastrin releasing peptide, NMC; neuromedin C, NMB; neuromedin B, NMU; neuromedin U, GAST; gastrin, CCK; cholecystokinin-8, SEC; secretin, SP; substance P, VIP; vasoactive intestinal polypeptide.

the final immunization. Therefore, the two antisera were characterized in the further study.

Characterization of bombesin antibodies

Specificity: The cross-reactivities of the antisera LBE 2G/2 and LBG 1G/2 to bombesin-like peptides or gastrointestinal peptides are shown in Fig. 1. The values of half-saturation concentration of each peptide to the bombesin antisera are also shown in Table 1. Both antisera LBE 2G/2 and LBG 1G/2 exhibited very high reactivity to synthetic bombesin with a half-saturation concentration of 16 and 27 pM, respectively. The LBE 2G/2 showed a

Table 1. Half-saturation concentrations of various bombesin-like peptides in binding of ^{125}I-[tyr^4]-bombesin to the bombesin antisera

Peptide	Half-saturation concentration, pM	
	LBE 2G/2	LBG 1G/2
bombesin	16	27
alytesin	345	22
litorin	562	$>10^6$
gastrin releasing peptide	53,400	22
neuromedin C	$>10^5$	38
neuromedin B	$>10^6$	$>10^6$
neuromedin U	$>10^6$	$>10^6$
ranatensin	22,200	$>10^5$

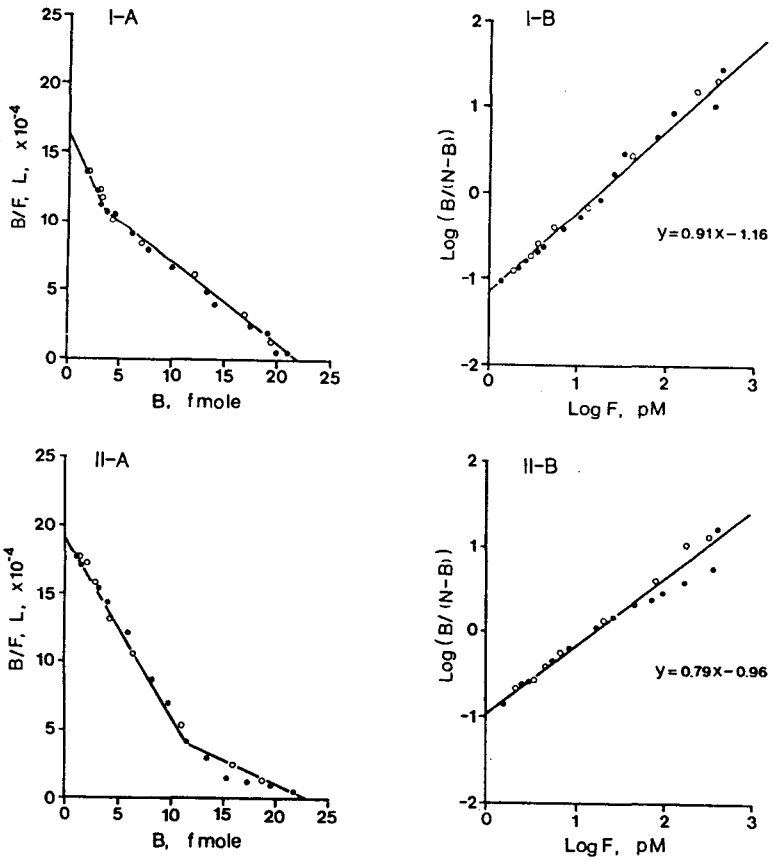

Fig. 2. Scatchard plots (A) and Sips plots (B) of labelled and unlabelled bombesin to bombesin antiserum, LBE 2G/2 (I), LBG 1G/2 (II). B and F indicate the bound and free bombesin concentration in titration, respectively. Filled circles represent data of titration with ^{125}I-[tyr^4]-bombesin while open circles represent those with unlabelled bombesin.

Table 2. Binding parameters of the bombesin antisera raised in guinea pigs

Binding parameter	LBE 2G/2	SBG 1G/2
Titer ($\times 10^3$)	188	43
Effective affinity constant (10^{11} M^{-1})	1.64	1.19
Average affinity constant (10^{11} M^{-1})	0.53	0.60
Total bombesin binding sites (μM)	4.66	0.97
Heterogeneity index (α)	0.91	0.79

very low cross-reactivity to other bombesin-like peptides except bombesin (Fig. 1A). The half-saturation concentrations of alytesin, litorin and gastrin releasing peptide (GRP) to the LBE 2G/2 were 345, 562 and 53,400 pM, respectively. The antiserum cross-reacted to neuromedin C, U and B much less than to GRP. On the other hand, the LBG 1G/2 exhibited high cross-reactivity to bombesin-like peptides (Fig. 1B). The half-saturation concentrations of alytesin, GRP and neuromedin C to LBG 1G/2 were 22, 22 and 38 pM, respectively. However, both antisera did not cross-react toward gastrointestinal peptides such as gastrin, secretin, cholecystokinin, vasoactive intestinal polypeptide (VIP) and substance P at concentrations as high as 10^{-7} M.

Affinity and Heterogeneity: The Scatchard plots and Sips plots of the two bombesin antisera are shown in Fig. 2 and the binding parameters of the antisera estimated from these plots are summarized in Table 2. The effective affinity constants of LBE 2G/2 and LBG 1G/2 were 1.64×10^{11} M^{-1} and 1.19×10^{11} M^{-1}, respectively. Both antisera exhibited curvilinear forms in the Scatchard plots, which indicates heterogeneity of the antibody population. The heterogeneity indices of LBE 2G/2 and LBG 1G/2 were 0.91 and 0.79, respectively. The total bombesin binding sites of LBE 2G/2 and LBG 1G/2 were 4.66 μM and 0.97 μM, respectively.

DISCUSSION

In the present investigation, two bombesin antisera distinct in terms of their specificity were successfully produced in guinea pigs by repeat injection of [lys^3]-bombesin conjugated to bovine serum albumin by different methods. [lys^3]-Bombesin, a synthetic bombesin in which an amino acid at the third position is substituted with lysine, is used in this study as in other studies (Ghatei et al, 1982; Major et al, 1983; Reeve et al, 1983). Synthetic bombesin seems not to be appropriate (data not shown) although carboxyl-terminal nonapeptide of bombesin has been used as an immunogen in other studies (Erspamer et al, 1979; Walsh et al, 1979).

The titer of antiserum LBE 2G/2 raised against [lys^3]-bombesin conjugated to bovine serum albumin with carbodiimide is 1:188,000 while that of antiserum LBG 1G/2 produced against [lys^3]-bombesin conjugated to bovine serum albumin with glutaraldehyde is 1 : 43,000. The titers of the bombesin antisera reported in literatures are in the range of 1: 1,000 (Walsh et al, 1979) and 1 : 640,000 (Major et al, 1983).

The antiserum LBE 2G/2 exhibits a very low cross-reactivity to all peptides tested in this study. Even bombesin-like peptides such as alytesin, litorin and GRP which have similar carboxyl-terminal heptapeptides to bombesin show a very high half-saturation concentrations to LBE 2G/2. On the other hand, the antiserum LBG 1G/2 exhibits a very high cross-reactivity to bombesin-like peptides including alytesin, GRP and neuromedin C but it reacts very weakly to peptides such as ranatensin, litorin and neuromedin B which are heterogenous in carboxyl-terminal amino acid sequence to bombesin. These results may suggest that LBE 2G/2 is specific for a region other than the carboxyl-terminus (probably amino-terminus) or complete 14 amino

acid sequence of bombesin whereas LBG 1G/2 is specific for the carboxyl-terminal region of bombesin.

The effective affinity constant (K_{eff}) of an antibody calculated from the initial slope of the Scatchard plot has been known as an important factor determining the sensitivity of radioimmunoassay (Ciabattoni, 1987). The K_{eff} values of LBE 2G/2 and LBG 1G/2 are $1.64 \times 10^{11}\ M^{-1}$ and $1.19 \times 10^{11}\ M^{-1}$, respectively. The two antisera produced in the present study exhibit a curvilinear pattern in the Scatchard plots, which indicates heterogeneity of the antibody population. It has been known that most polyclonal antibodies are usually heterogenous with respect to affinity for the antigen (Berson & Yallow, 1959; Karush, 1962). The indices of heterogeneity (α) calculated from the Sips plot of LBE 2G/2 and LBG 1G/2 are 0.91 and 0.79, respectively. A high value of the hetrogeneity index does not necessarily indicate that the antibody is structurally homogeneous but only suggests that the antibody is monodisperse with respect to the binding affinity (Haber et al, 1967). It has been generally accepted that an antiserum of which K_{eff} is $>10^{11}\ M^{-1}$ and α is >0.5 is indispensable for the highly sensitive radioimmunoassay (Rehfeld et al, 1972; Chang & Chey, 1980). From this point of view, the bombesin antisera raised in this study is supposed to be sufficient for a very sensitive radioimmunoassay of bombesin.

It is concluded from the above results that the antisera produced in this study exhibit the sufficient titer, affinity and heterogeneity index which are essential for a highly sensitive radioimmunoassay. The antiserum LBE 2G/2, raised against [lys^3]-bombesin conjugated to bovine serum albumin with carbodiimide, is an extremely specific antiserum which can be used for a very sensitive radioimmunoassay of bombesin, as it has very low cross-reactivity to all bombesin-like peptides. The antiserum LBG 1G/2, produced against [lys^3]-bombesin conjugated to bovine serum albumin with glutaraldehyde, is an antiserum which could be used for determination of bombesin-like petides such as alytesin, GRP and neuromedin C when differentiation of the peptides is not necessary.

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