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Bioeffective monoclonal antibody against the decapeptide gonadotropin-releasing hormone: Reacting determinant and action on ovulation and estrus suppression

(reacting epitopes/ovulation block)

G. P. TALWAR, S. K. GUPTA, VINOD SINGH, DINKAR SAHAL, K. S. N. IYER, AND OM SINGH

National Institute of Immunology, Post Box No. 4922, New Delhi-110 029, India

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ABSTRACT A monoclonal antibody generated against the decapeptide gonadotropin-releasing hormone (GnRH) was effective in intercepting the bioactivity of the hormone; it blocked ovulation in rats. The antibody reacted optimally with the native hormone. Substitution of amide at the COOH terminus by a carboxyl group decreased immunoreactivity by a factor of 200. The antibody recognized the amino acid sequences 4–6, 7–10, and 4–10 to a variable degree, which suggests that the epitope has a conformation involving the entire molecule, with the NH_2- and COOH-terminal regions probably in proximity. The antibody was also competent to suppress the progression of estrus in dogs, an indication that GnRH may play an inductive role in the reproductive function of dogs.

Gonadotropin-releasing hormone (GnRH) has a pivotal role in mammalian reproduction. By regulating the secretion of follicle-stimulating hormone and luteinizing hormone from the pituitary, it controls spermatogenesis in the male and ovulation in the female, with concomitant production of sex steroids. The hormone is a decapeptide, which has been synthesized along with several analogs having agonistic or antagonistic properties pointing to the portions of the molecule important for the biological activity of the hormone (1, 2). Attempts have also been made to map the reading framework of the conventional polyvalent antibodies raised against the hormone (3, 4). This communication reports the bioefficacy of a monoclonal antibody generated against the hormone for block of ovulation in rats. The binding characteristics of this antibody with the entire decapeptide and subpeptide fragments have been explored to obtain information on the topology of the epitope(s) important for bioneutralization. A practical use of the antibody is indicated by its ability to suppress the progression of estrus in dogs.

MATERIALS AND METHODS

Synthetic GnRH (lot IBR5483) was made available by the Population Council, New York (courtesy Harold Nash and T. Jackanicz), and by the Center for Population Research, National Institute of Child Health and Human Development, National Institutes of Health (courtesy G. Bialy).

Synthesis of GnRH Fragments. The tripeptide (amino acids 4–6), tetrapeptide (amino acids 7–10), and heptapeptide (amino acids 4–10) of GnRH were synthesized by classical solution phase methodology. The α-amino group of amino acids was protected by *t*-butyloxycarbonyl (Boc). The hydroxyl group of serine and tyrosine were protected by benzyl groups. The guanidine of the arginine was protected by a nitro group. Coupling was carried out by the dicylohexyl carbodiimide-1-hydroxybenzotriazole method. The final condensation of tripeptide, Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OBzl with tetrapeptide, H-Leu-Arg(NO_2)-Pro-Gly-OBzl, was carried out by the azide procedure (5). The removal of protection from the Boc group and the nitro and benzyl groups was done by anhydrous trifluoracetic acid and catalytic hydrogenolysis, respectively.

Monoclonal Antibody. A number of mouse hybrid cell clones secreting antibodies binding to GnRH were developed as described (6). The monoclonal antibody used in this study was the secretory product of hybrid cell clone $P_8 16_{62}$. Hybrid cells were grown as ascites in the intraperitoneal (i.p.) cavity of BALB/c mice primed with Pristane (Aldrich). Ascites fluid tapped from the i.p. cavity was made cell-free by centrifugation at 800 × *g* for 15 min at 4°C and then heat-inactivated at 56°C for 30 min and centrifuged at 15,000 × *g* to remove debris. This ascites fluid, used as a source of antibody, had a titer of 10^6 (i.e., 30%–40% binding of ^{125}I-labeled GnRH at 10^6 dilution) in RIA. The antibody was specific to GnRH. It was devoid of reactivity with thyrotropin-releasing hormone in competitive RIA. The cross-reactivity (based on ED_{50}) with GnRH analogs D-Ser $(Bu^t)^6$-des-Gly^{10}-GnRH ethylamide and Benz-His^6-GnRH was lower than with the native hormone by factors of 387 and 608 (7). The association constant (K_a) for binding with GnRH was 1.2×10^9 liters/mol as calculated by Scatchard plot (7). The antibodies were of IgG_{2a} class with κ light chain.

Radioimmunoassay. The reactivity of the hybrid cell product with various peptides was determined by competitive displacement studies in the RIA using ^{125}I-labeled GnRH and antibody of appropriate dilution to obtain 30%–40% binding in the absence of competing peptide. Iodination of GnRH (2 μg) with 1 mCi of carrier-free $Na^{125}I$ (1 Ci = 37 GBq) (Amersham) was carried out as described (3). Over 80% of the labeled hormone was bound in the presence of excess antibody. The range of specific activity of ^{125}I-labeled GnRH was 1400–1600 μCi/μg. Bound ^{125}I-labeled GnRH was separated by the method of Jeffcoate *et al.* (8).

Measurement of Heterogeneity Index of the Clonal Product. The heterogeneity index (a) was calculated by using logarithmic transformation of the Sips equation (9)

$$\log \frac{B}{Ab_t - B} = a \log K + a \log F$$

where B is bound hormone, F is free hormone concentration, and Ab_t is antibody binding sites. A plot of $\log B/(Ab_t - B)$ versus $\log F$ gave a straight line with a slope of heterogeneity index a; a has a value of 1 for homogeneous or single antibody population with respect to antigen-binding affinity.

Ovulation Inhibition. Adult female rats (Holtzman strain), weighing 200–300 g, were observed for regular cyclicity over 2–3 consecutive estrus cycles and were given 0.5 ml i.p. of the ascites fluid containing anti-GnRH on the morning (9:00

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Abbreviation: GnRH, gonadotropin-releasing hormone.

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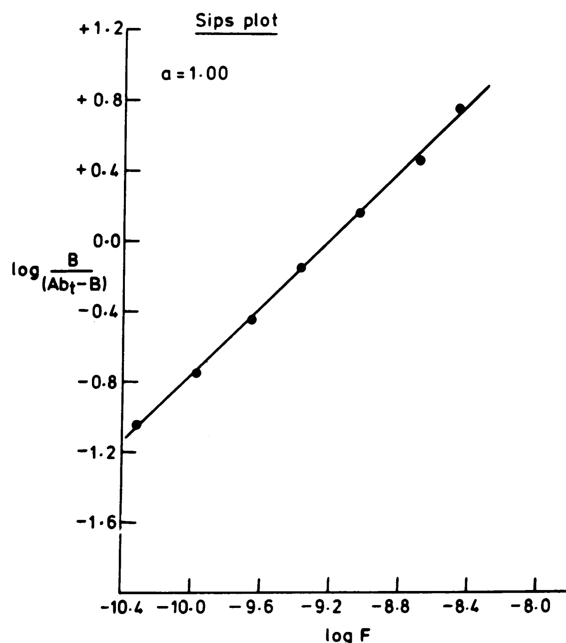

FIG. 1. Plot of log $(B)/(Ab_t - B)$ vs. log F for heterogeneity index, a, of the product of clone $P_8 16_{62}$.

a.m.) of proestrus. Control rats received an equivalent volume of ascites obtained by growing SP2/0 mouse myeloma cells. Animals were autopsied the following day (day of estrus), and oviducts were flushed for counting of eggs.

Block of Estrus in Female Dogs. Female dogs of mixed breed were procured locally, dewormed, and kept under observation in the Institute Animal House. Estrus and its progression were monitored according to the following criteria; attraction of male, vaginal swelling, cytology, and receptivity of the female. Presence of rounded epithelial cells in vaginal smears was interpreted to indicate the proestrus. Estradiol and progesterone in circulation were determined by RIA (10). Anti-GnRH antibody in ascites fluid, after sterilization by passage through a Millipore filter (pore size, 0.45 μm), was injected intravenously in a single dose of 3.0 ml in dogs at proestrus.

RESULTS

Evidence for the Monoclonal Nature of the Antibody. The product of hybrid cell clone $P_8 16_{62}$ was composed of a homogeneous population of antibody. The heterogeneity index, a, was 1, a result confirming the monoclonal nature of the secreted product (Fig. 1).

Bioefficacy of the Monoclonal Antibody. Antibodies against peptide or steroid hormones are not necessarily of the bioneutralizing type. Failure of antibodies, otherwise immunoreactive in RIA, to inhibit the biological action of the hormone *in vivo* has been reported for antibodies against carboxyl-terminal peptides of β-hCG (human chorionic gonadotropin) (11, 12), β-TSH (thyrotropic hormone) (13), and steroids (14). It was, therefore, important to find out

Table 1. Effect of passive administration of monoclonal anti-GnRH antibody on ovulation in proestrus rats

Treatment	No. ovulating/ total	No. of ova (mean ± SEM)
Control	6/6	5.8 ± 0.5
$P_8 16_{62}$	0/6	0

Ascites fluid (0.5 ml) of clone $P_8 16_{62}$ was injected i.p. at 9:00 a.m. on proestrus. Control group received an equivalent amount of ascites fluid devoid of anti-GnRH antibody.

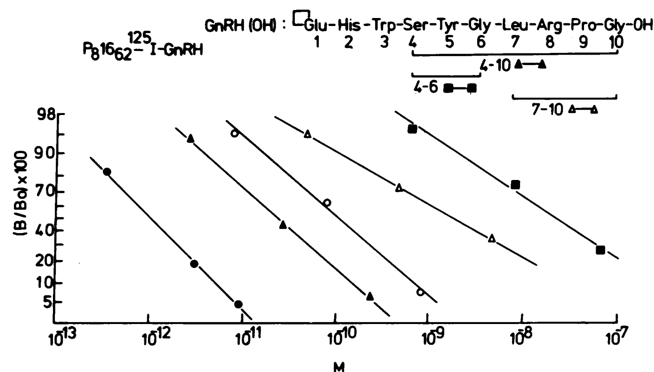

FIG. 2. Competition for binding of ^{125}I-labeled GnRH(NH_2) with monoclonal antibody $P_8 16_{62}$ by GnRH(NH_2) (●), GnRH(OH) (○), GnRH(OH) (amino acids 4–10) (▲), GnRH(OH) (amino acids 7–10) (△), and GnRH(OH) (amino acids 4–6) (■) in RIA. B, radioactivity bound in presence of labeled GnRH and competing unlabeled peptide. Bo, radioactivity bound with labeled GnRH alone. Values are expressed as molarity.

whether this monoclonal antibody, directed against a short peptide, having primarily a single site for binding with the peptide, had the ability to intercept the biological action of GnRH. Ovulation being dependent on the luteinizing hormone surge induced by GnRH, the effect on ovulation in rats was investigated. The anti-GnRH monoclonal antibody given at proestrus blocked ovulation in all six test animals (Table 1).

Binding Characteristics of the Monoclonal Antibody. The antibody bound best with the native hormone (glycine amide). The analog with the terminal glycine-free carboxyl retained reactivity with the antibody, but the competition was markedly lower than with the native hormone having amide at the COOH-terminus. Competition by the tetrapeptide (Leu-Arg-Pro-Gly—i.e., amino acids 7–10) and tripeptide (Ser-Tyr-Gly—i.e., amino acids 4–6) with iodinated native GnRH for binding to the antibody was poor. The heptapeptide reacted better with both of the above-mentioned sequences than did the individual peptides (Fig. 2). The amounts of the different peptides needed for achieving 50% inhibition of binding of native GnRH to monoclonal antibody are given in Table 2. These results indicate the optimal binding of the antibody with the entire molecule and suggest that the reactive topology is contributed to by both the NH_2- and COOH-terminal parts. The proximity of the NH_2 terminus to the COOH terminus of the decapeptide has been indicated in spectroscopic (15) and computer graphic studies (16).

Suppression of Estrus in Dogs. Dogs usually experience estrus twice a year. Information on the endocrinology of dogs is scanty, and formal evidence is lacking for the presence or role of GnRH in induction of estrus in the species. Because

Table 2. Amount of peptide needed for 50% inhibition of binding of ^{125}I-labeled GnRH to monoclonal antibody $P_8 16_{62}$

	ED_{50}, M	Relative reactivity $\times 10^3$
GnRH(NH_2)	1.0×10^{-12}	1000.00
GnRH(OH) (amino acids 1–10)	1.0×10^{-10}	10.00
GnRH(OH) (amino acids 4–10)	2.3×10^{-11}	43.60
GnRH(OH) (amino acids 7–10)	1.7×10^{-9}	0.58
GnRH(OH) (amino acids 4–6)	2.1×10^{-8}	0.05

ED_{50}, median effective dose.

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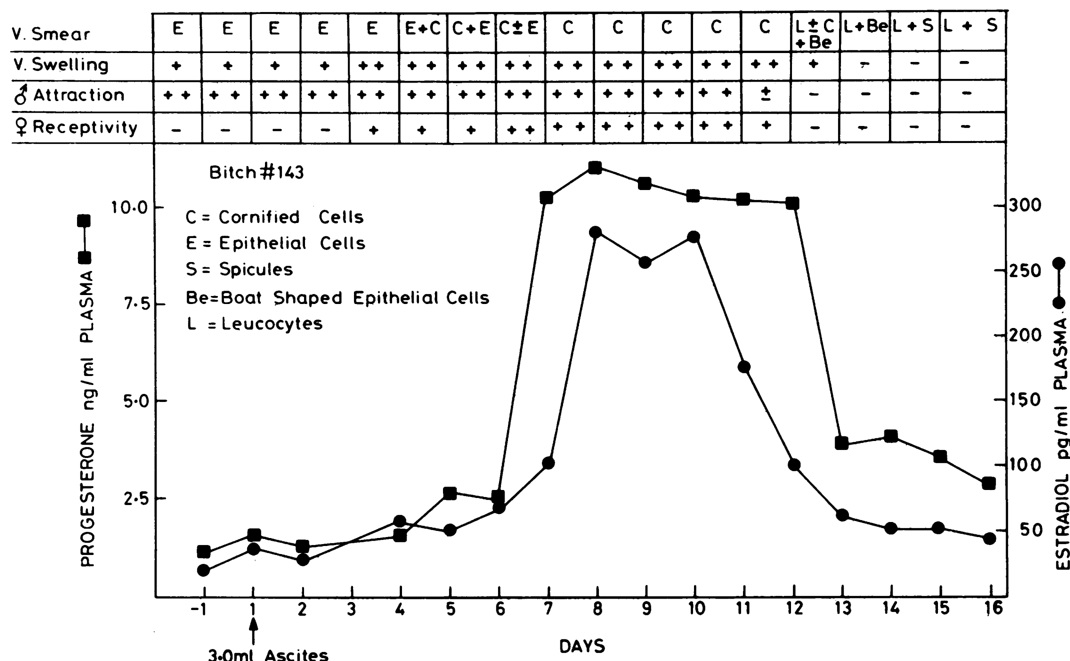

FIG. 3. Normal progression of estrus in female dog treated i.v. with 3 ml of the ascites fluid obtained with mouse myeloma cells (SP2/0) and devoid of anti-GnRH antibodies.

GnRH does not circulate in amounts high enough to permit direct assay, even in the mammals in which its role is known, estimation of the blood level of the hormone in the dogs by direct RIA was not feasible. Instead, the effect of the monoclonal anti-GnRH antibody on estrus was tested in female dogs given a single intravenous injection of 3.0 ml at proestrus.

Fig. 3 illustrates the biological profile during heat of a female dog that received control ascites fluid obtained by growing SP2/0 mouse myeloma cells. Fig. 4 typifies the pattern observed in dogs injected with ascites fluid containing anti-GnRH antibody. Within 48 hr, there was a decrease both in the receptivity of the female to the male and in the male's attraction to the female. None of the females proceeded to estrus; leukocytes were detected within 24–48 hr. No increase in blood progesterone or estradiol was noted in the animals receiving anti-GnRH, in contrast to the hormonal changes of the control animal approaching estrus (Fig. 3). The antibody was measurable in circulation for >14 days, although titers decreased progressively.

DISCUSSION

Interference of the bioactivity of a hormone by antibody demands the prevention by antibody of the binding of hormone

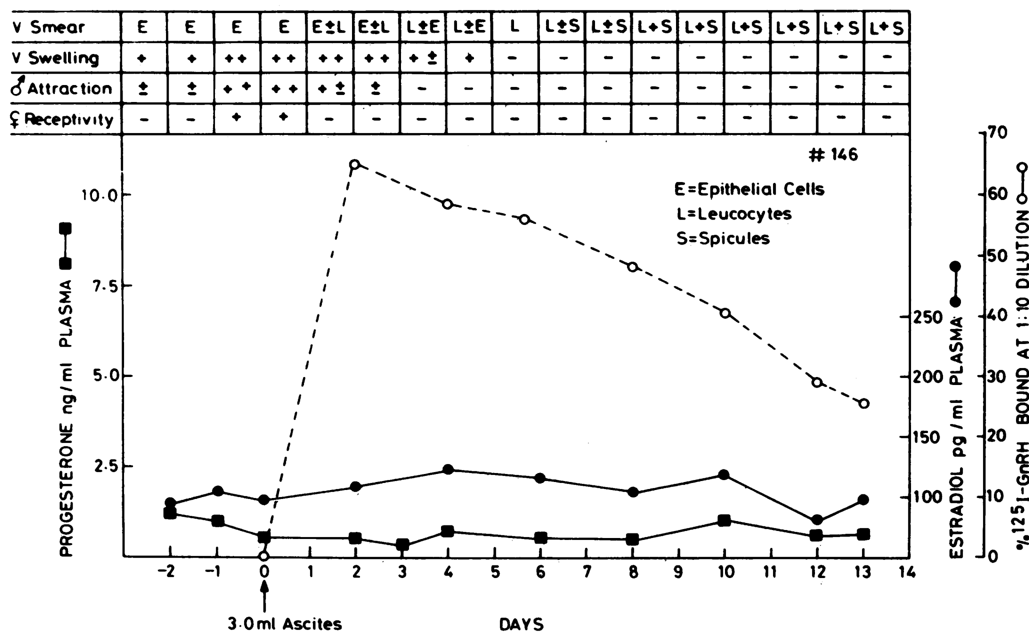

FIG. 4. Typical effect of administered anti-GnRH monoclonal antibody given to female dogs in the proestrus stage. The antibody suppressed heat within 48 hr as determined by the four criteria listed in the upper part of the figure. The increase in blood progesterone and estradiol normally occurring during estrus was also prevented. Dashed line shows amount of antibody in circulation on different days after a single i.v. injection of 3 ml of the ascites fluid of clone $P_8 16_{62}$.

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to the receptor and the subsequent triggering action of the hormone on the target cell. To achieve this, antibody must be directed either against the part of the hormone molecule binding to the receptor or elsewhere in a manner producing stereochemical constraints on the interaction of the hormone with the tissue receptors. In situations in which the antibody fails to impede the hormonal activity in spite of its immunoreactivity, as measured by RIA (11–14), it can be speculated that the antibody is bound to the hormone in a manner distinct from the requirements for bioneutralization. The low avidity of the antibodies may also be responsible for incomplete scavenging of the hormone as an immune complex (17). Polyvalent antibodies would, thus, normally have better properties of bioneutralization of hormonal bioactivity. The present antibody, which is defined as a monoclonal antibody, not only on the grounds of its repeated cloning by limiting dilution but also by its single isotype of heavy and light chain and the physicochemical properties of monolinear association constant, demonstrated the exceptional merits of intercepting the hormonal activity of GnRH *in vivo*. The antibody is directed against a conformation in which both the NH_2- and COOH-terminal regions of GnRH participate, a postulate consistent with data based on physicochemical and computer graphic studies (15, 16).

The monoclonal antibodies were effective in blocking ovulation in rats as well as in suppressing the progression of estrus in female dogs, as indicated by behavior changes, vaginal cytology, and sex hormonal profiles. These experiments provide indirect evidence for the involvement of the GnRH in induction of estrus in this species. Although these antibodies have been used in female animals in the present study, it can be assumed that they will similarly be active in the male in blocking events dependent on GnRH.

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