J. steroid Biochem. Vol. 23, No. 5B, pp. 795–800, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

IMMUNOBIOLOGY OF GONADOTROPIN-RELEASING HORMONE

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Summary—This article will review methods successful in inducing antibody responses against gonadotropin releasing hormone without the use of Freund's complete adjuvant (FCA), the characteristics of the antibodies produced, and will describe the dominant antigenic determinant(s) of the decapeptide and the use of monoclonal antibodies for suppression of estrus and of sex steroid production. The potential application of such immunological approaches in veterinary species, in management of precocious puberty in humans, and in hormone dependent cancers, is indicated.

INTRODUCTION

The decapeptide gonadotropin releasing hormone (GnRH), a "unisex" molecule has a pivotal role in control of both male and female fertility. Although data will be discussed in this paper on the presence and the likely role of this hormone in gonads and other extra-hypothalamic tissues, there is ample evidence that this hormone has a distinct physiological function in the hypothalamic–pituitary axis. It is one of those molecules which has been conserved through evolution. The primary sequence of the hormone from ovine and porcine sources is identical and the synthetic peptide based on this sequence is biologically active in most mammals tested so far including the humans [1, 2].

Antibodies specific to the hormone and capable of neutralizing its activity can constitute agents for interception of the hormonal action. They can provide evidence for the location of the hormone in expected or suspected tissues. Cells engaged in its synthesis and secretion can be identified. The binding of the hormone to receptor bearing cells and the disappearance of the hormone–receptor complexes in course of time can be followed up by sequential immunolocalization studies. Antibodies are therefore useful agents not only for interference in the action of the hormone and thereby the block of fertility, but also for various studies connected with the mechanism of the action of the hormone.

INDUCTION OF ANTIBODY RESPONSE

Several attempts have been made in earlier years to raise antibodies against this decapeptide by absorbing it either on alhydrogel [3, 4], charcoal [5] or polyvinylpyrrolidone (PVP) [6–8]. These methods required the use of FCA. Moreover, high antibody titres were not obtained. An approach which has been employed by many investigators in recent years is the use of carriers. The peptide is linked to a "foreign" or non-self protein to mobilize helper "T" cell function. However, most of the work reported in literature even with carriers has been carried out using FCA. One can visualize that for any practical application, a modality of immunization without FCA has to be evolved.

An alternate scheme, namely of linking the GnRH molecule through a short bridge to an adjuvant, the muramyl dipeptide (MDP) to generate an immunogenic complex has been reported by the Institut Pasteur group headed by Louis Chedid[9]. In the near future data should be available from other laboratories to indicate whether this conjugate is workable. Another totally synthetic peptide of 16 aminoacids, ten of GnRH, a bridge of 2 aminoacids and a tetrapeptide adjuvant, Tuftsin, has been made by one of my co-workers Dr Manas Choudhuri. This is under evaluation. Another peptide of 30 aminoacids consisting of GnRH, an immunodominant epitope of Hepatitis-B and the tetrapeptide Tuftsin is being synthesized in our laboratory. Conceptually this peptide would have 3 moieties: the hormone, a carrier and an adjuvant.

At present the best tested approach to evoke the formation of anti-GnRH antibodies is to use the hormone linked to a carrier protein. The most frequently used carriers has been the bovine serum albumin (BSA) [6, 7, 10–19], but several other proteins have also been used, such as human serum albumin (HSA) [7, 20], thyroglobulin [15, 20, 21], tetanus toxoid (TT) [22–24], horse radish peroxidase (HRPO) [25], human lactoferrin [26] key-hole limpet haemocyanin (KLH) [28], guinea pig γ-globulin [27].

The carrier can be conjugated at several points in the molecule; it can be attached at the hydroxyl (OH) of serine or tyrosine directly or via, a "bridge" introduced by derivatization of GnRH [5, 11, 12 and 21]. It can be inserted at the C-terminus when the synthesized decapeptide has free —COOH group of gly^{10}. One can also link the carrier at *N*-terminus by opening out the pyroglutamyl end. Figure 1 indicates the possible sites of linkage of GnRH with carrier proteins. Of course, the properties of conjugates thus made would differ.

The nature of the carrier influences the immunogenecity of the conjugates. Some time ago in our laboratory Nilabh Shastri carried out studies on the

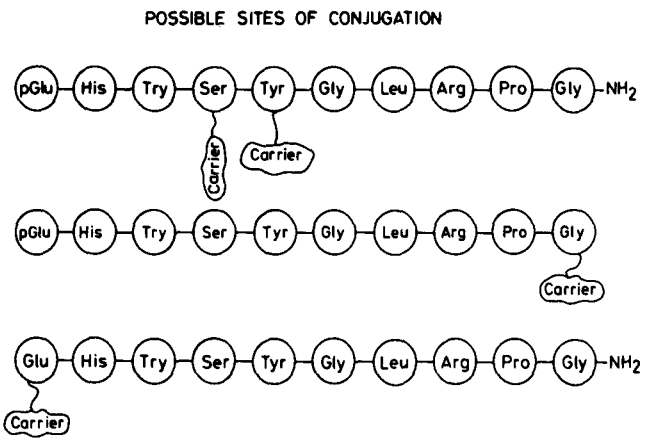

Fig. 1. Structure of Gonadotropin-releasing hormone and possible sites of linkage of carrier proteins.

relative merits of three carriers. BSA, a frequently used carrier in past was a poor immunogen in mice if used without FCA. However, antibodies were obtained if it was adsorbed on alum and given with detoxified derivative of lipopolysaccharide, SPLPS [28]. Two other carriers were employed namely tetanus toxoid (TT) and KLH which were much better (Fig. 2). The molar ratio of the peptide to the carrier was also a factor influencing the overall immunogenecity. In these studies, TT appeared to be a superior carrier for conjugation to GnRH.

It is indeed possible to produce antibodies against GnRH using TT as carrier, without the use of FCA. For bioefficacy adequate titres of the antibody are required. Moreover, the response should be of sufficiently long duration. Figure 3 gives antibody titres in a female bonnet monkey immunized initially with three injections of GnRH-TT with muramyl dipeptide analogue 1 [2-(2-hexamido-2-deoxy-D-glucose-3-oxyl)-D-propionyl-L-ananyl-D-isoglutamine] and alum. A booster injection of GnRH-TT with an innocuous preparation a lipidic emulsion LBA (Leiras Basic Adjuvant available from Leiras Pharmaceutical Turku, Finland) was given which produced high enough antibody titres to interfere in the normal cyclicity of the animal. The duration of the response was fairly long [29]. We have similar data on a baboon whose fertility and cyclicity was blocked for 160 weeks by immunization with GnRH-TT given with SPLPS and alum as adjuvants in primary immunization followed by a booster with LBA. The antibody titres in this baboon are now declining. The sex steroid levels were measured in these animals at various time points and as expected, these were very low (Luteal Progesterone = <0.3 μg/ml plasma).

BINDING CHARACTERISTICS OF THE ANTIBODIES; THE NATURE OF ANTIGENIC DETERMINANT OF GnRH

Investigations were carried out to determine the part of the molecule or the epitope recognized by the antibodies obtained by the above mentioned immunization procedure. Antibodies from both the monkey and the baboon in whom the response was of long duration and clearly bioeffective, had some similarities. In both cases the antibodies were of high affinity, the K_a was of the order of 10^{10} to 10^{11} l/mol. The antibodies were analysed for binding with the native hormone and a number of sub-peptides. They reacted maximally with the native GnRH(NH_2) but what is interesting is, they did not react at all with the GnRH free acid. A minor change at the C-terminus

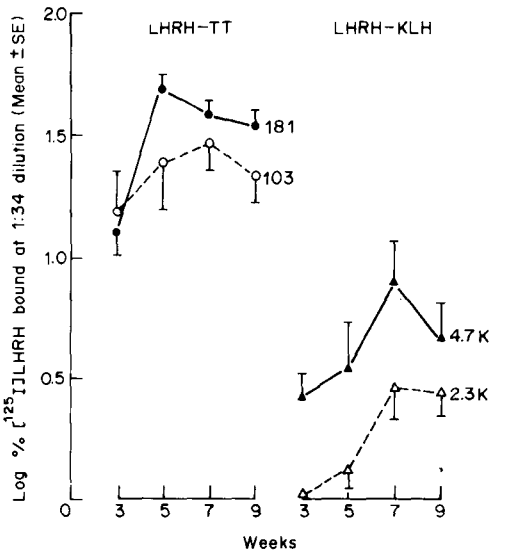

Fig. 2. Anti-GnRH response in mice immunized with GnRH–TT and GnRh–KLH conjugates. Two conjugates of GnRH with each carrier differing in molar proportions of GnRH per mole of the carrier were used as indicated. Immunization was carried out in ten mice in each group. Three injections were given, each containing 4 μg of carrier conjugated GnRH adsorbed on alum. Points represent mean titres ± standard error as a function of time after immunization. Titres are given on a logarithmic scale. K stands for 1000. Results are expressed as percent radioactivity bound at the indicated dilution of serum after substracting non-specific binding (4–6%) with normal mouse sera at the same dilution [Data from reference 28].

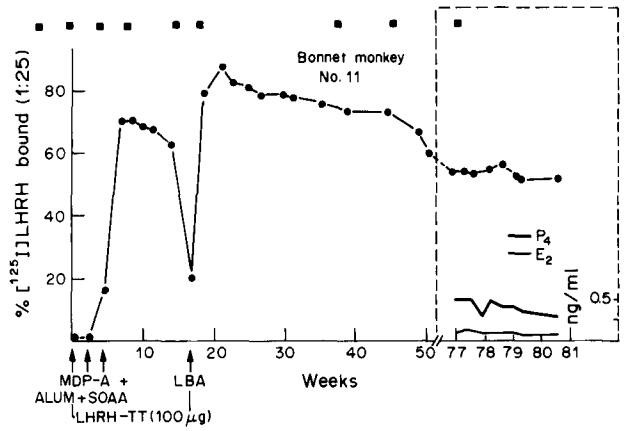

Fig. 3. Active immunization against GnRH in a bonnet monkey. The animal received three injections of GnRH–TT with the indicated adjuvants and a single secondary immunization with an innocuous adjuvant, Leiras basic adjuvant consisting of an emulsion of metabolizable lipids. The cyclicity of the animal depicted by the bleeding days (black squares on the upper part of the figure) was disrupted during the period of high antibody titres. The plasma progesterone and estrogens were also low in one of the cycles shown in the figure [Data from reference 29].

e.g. replacement of glycine amide by acid group (—COOH) abolished totally the recognition. These antibodies did not bind with the synthetic fragments of GnRH, the tripeptide (aminoacid 4–6), the tetrapeptide (aminoacid 7–10) and the heptapeptide (aminoacid 4–10). Thus, these antibodies can be conceived to be essentially conformation reading where conformation peculiar to the total native GnRH is read. Instances have been reported in the literature where antibodies recognised the C-terminus [3, 5, 7, 15, 21, 30], *N*-terminus [7] or sequences here or there between various subparts of the molecule [3, 15] besides the total conformation.

It is not necessary that the bioeffective antibody be only the one which recognizes the total GnRH and not the subparts of GnRH. The characteristics of a monoclonal developed in our laboratory is an example [31]. This monoclonal reacts maximally with GnRH(NH_2) but also with GnRH(OH), though the recognition of the latter was diminished by 100 times [32]. It also reacted with several synthetic fragments of GnRH, suggesting that it recognizes nearly all parts of the molecule to a lesser or greater extent. Table 1 and Fig. 4 give data on its characteristics. The immunoreactivity profile of this monoclonal antibody is suggestive of an all embracing epitope constituted by the proximation of the amino and C-terminus parts of GnRH. Preliminary computer graphic studies of Feldman at NIH are consistent with this view. This is also in good agreement with the structure proposed by Momany on the basis of empirical energy calculations [33]. These studies point to a conformation of GnRH where *p*Glu^1 and Gly-NH_2^{10} are close to each other.

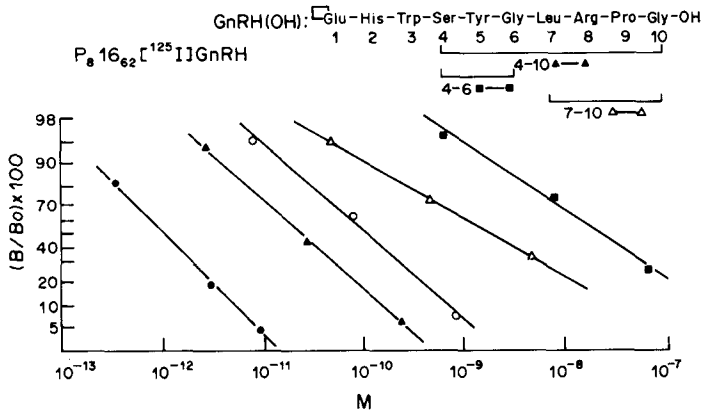

Fig. 4. Competition for binding of ^{125}I-labelled GnRH(NH_2) with monoclonal antibody $P_8 16_{62}$ by GnRH(NH_2) (●——●), GnRH(OH) (○——○); GnRH(OH) (amino acid 4–10) (▲——▲), GnRH(OH) (amino acid 7–10) (△——△) and GnRH(OH) amino acid (4–6) (■——■) in RIA. B, radioactivity bound in presence of labelled GnRH and competing unlabelled peptide. Bo, radioactivity bound with labelled GnRH alone. Values are expressed as molarity (Data from reference [32]).

Table 1. Amount of peptide needed for 50% inhibition of binding of [^{125}I]GnRH to monoclonal antibody $P_8 16_{62}$

	ED_{50} M	Relative reactivity × 10^3
GnRH(NH_2)	1.0×10^{-12}	100.00
GnRH(OH) (aminoacid 1–10)	1.0×10^{-10}	10.00
GnRH(OH) (aminoacid 4–10)	2.3×10^{-11}	43.60
GnRH(OH) (aminoacid 7–10)	1.7×10^{-9}	0.58
GnRH(OH) (aminoacid 4–6)	2.1×10^{-8}	0.05

ED_{50}, median effective dose.
Data from Reference [32].

BIOLOGICAL EFFECTS OF ANTIBODIES AGAINST GnRH

A number of mouse hybrid cell clones secreting anti-GnRH antibodies were developed as described [31]. The monoclonal antibody used in the following experiments was the secretory product of hybrid cell clone $P_8 16_{62}$. Hybrid cells were grown as ascites in the intraperitoneal cavity of BALB/c mice. The ascitic fluid, used a source of antibody, had a titre of 10^6 (i.e. 30–40% binding of ^{125}I-labelled GnRH at 10^6 dilution in radioimmunoassay (RIA). The antibody was specific to GnRH and 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 Bzl-His^6-GnRH was lower than with the native hormone by a factor of 387 and 608 [34]. The association constant (K_a) was 1.2×10^9 l/mol as calculated by Scatchard plot. A single injection of this monoclonal antibody given to mice produced a complete block of cyclicity, and the number of cycles suppressed were proportional to the amount of antibody injected [34]. In another experiment, the ascitic fluid containing anti-GnRH antibody was given to rats at proestrus stage. The treatment inhibited completely ovulation in rats [32]. These properties of the monoclonal antibodies are suggestive of potential veterinary applications. One of the applications can be in a species where ovulation is periodic e.g. the female dogs. A single injection of the monoclonal antibody caused suppression of estrus in bitches as measured by different parameters (Fig. 5). The control experiment illustrates the normal progression of estrus in the species as assessed by behaviour, vaginal swelling, cytology and steroid hormone profiles (Fig. 6).

The above mentioned effects of anti-GnRH antibodies on block of ovulation and cyclicity are explainable at the level of hypothalamic–pituitary interaction. Described below is an experiment which may fit in the same mode of GnRH action or may have an additional explanation. Mice were rendered pregnant and pregnancy confirmed by mini-leprotomy. Anti-GnRH antibodies were given on any time between day 7–9 of pregnancy which resulted in resorption and termination of pregnancy. The effect of antibodies could be rescued by progesterone [34]. The antibodies could have blocked gonadotropin production from the pituitary which in turn induce the production of progesterone from the ovaries. Whether progesterone is contributed by foeto–placental unit in this species is not clearly delineated. The presence of GnRH in human placenta has been demonstrated by a number of investigators [35–37]. Recent experiments in our laboratory have also indicated the enhanced secretion of human chorionic gonadotropin (hCG) by chorionic villi *in vitro* in response to GnRH. Addition of antibodies in the medium prevented the GnRH induced secretion of hCG. Thus a second site of the action of GnRH and

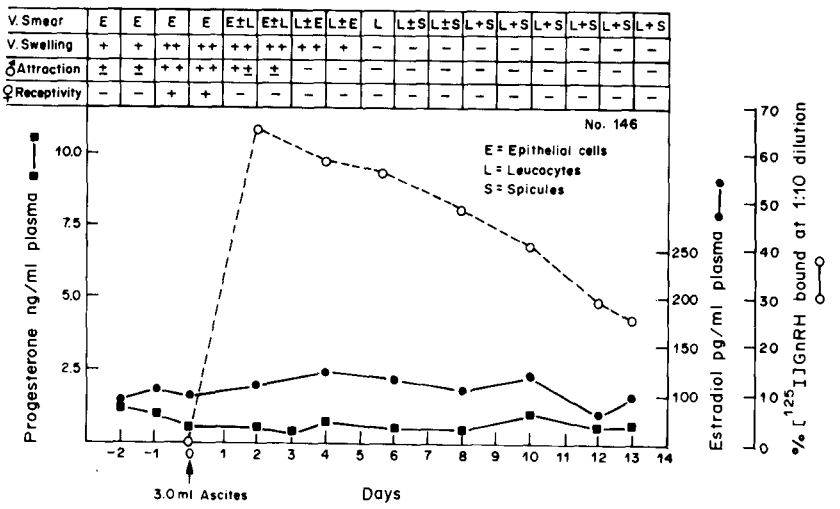

Fig. 5. Typical effect of anti-GnRH monoclonal antibody administered to female dogs at the proestrus stage. The antibody suppressed heat within 48 h as determined by the four criteria listed in the upper part of the figure. The increase in blood progesterone and estradiol normally occurring during estrus was also prevented. Dotted line shows the amount of antibody in circulation on different days after a single i.v. injection of 3 ml of the ascites fluid of clone $P_8 16_{62}$ (Data from reference [32]).

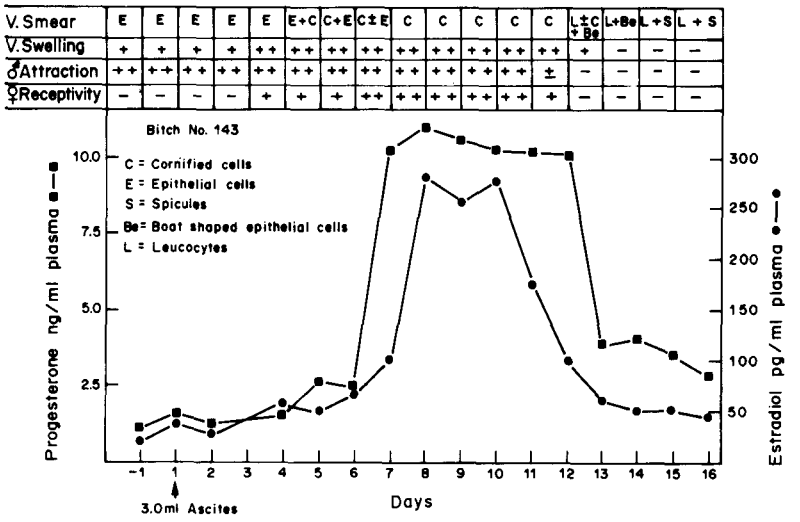

Fig. 6. Normal progression of estrus in female dog given 3.0 ml of ascites fluid obtained with mouse myeloma cells (SP2/0) and devoid of anti-GnRH antibodies (Data from reference [32]).

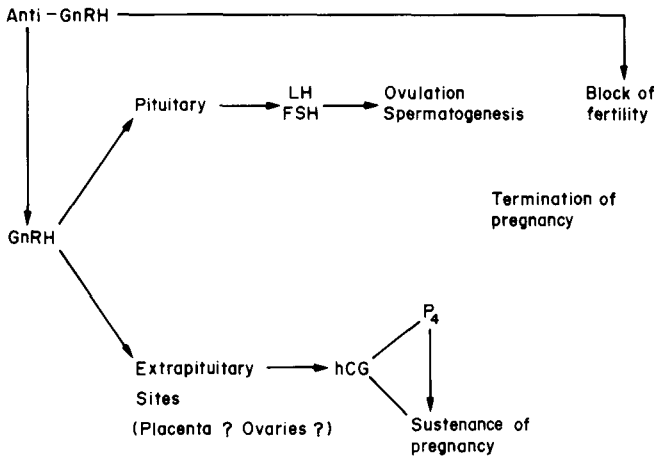

Fig. 7. Two potential sites of action of anti-GnRH antibodies mammalian reproductive system.

its antibodies can be hypothesized namely the interception of GnRH stimulated chorionic gonadotropin in placenta (Fig. 7).

Acknowledgements—This work benefitted from research grants of the Rockefeller Foundation, the National Co-ordinated Project of the Indian Council of Medical Research and the Indo–U.S. Collaborative Programmes.

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