PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 643-646 (1978)

Development of a GnRH Radioimmunoassay Utilizing a Superactive Synthetic GnRH Analog: D-[Lys⁶]-GnRH (40264)

D. HEBER AND W. D. ODELL

Departments of Medicine and Physiology, UCLA School of Medicine, and Division of Endocrinology and Metabolism, Harbor General Hospital Campus, Torrance, California 90509

Since the sequencing and synthesis of the hypothalamic decapeptide gonadotropin releasing hormone, GnRH (1, 2), a number of laboratories have developed radioimmunoassays (RIA's) said to be both sensitive and specific for GnRH (3–8). This decapeptide was immunogenic when injected into animals in unconjugated form, but the antibodies obtained were of low titer and sensitivity (4). Conjugation to a larger protein has been widely used to produce high affinity antibodies in high titer to peptides with molecular weight less than 4000 (10). Since GnRH has neither a free carboxy or amino terminus it has been coupled to larger proteins by use of a bifunctional reagent, bis-diazotized benzidine (3) as was first done for thyrotropin releasing hormone (11). The use of a bifunctional reagent may result in peptide dimerization. To avoid this Koch *et al.* (7) developed a two-step conjugation in which *p*-diazonium phenylacetic acid was attached to GnRH and the resulting diazo derivative coupled to carrier protein using carbodiimide. Both methods yield antisera recognizing both the carboxy and amino termini of GnRH, although the N-terminal specificity is somewhat less for the latter antiserum. Twenty percent cross-reaction with des-[pGlu^{1}]-GnRH is observed with this antiserum compared to 3.8% for that obtained using bis-diazotized benzidine (3).

In an attempt to obtain an antiserum recognizing both the C- and N-termini of GnRH which might recognize GnRH differently than the available antisera, we investigated methods of conjugating at the number 6 glycine position. The biologically superactive synthetic analog D-[Lys^{6}]-GnRH provided an ϵ-amino group three carbons removed from the GnRH peptide chain (see Fig. 1). This analog is also resistant to enzymatic degradation which occurs at the number six to seven position (12). Moreover, conjugation to a larger protein without peptide dimerization is theoretically possible for this synthetic analog. A GnRH RIA was developed and characterized using D-[Lys^{6}]-GnRH both to simplify production of high affinity antisera to GnRH in a single step reaction and in hopes of producing an antibody more specific for native GnRH.

Materials and methods. Conjugation. D-[Lys^{6}]-GnRH (Bachem, Inc., Torrance) was conjugated to thyroglobulin using carbodiimide as previously described by Skowsky *et al.* (10) using a peptide: thyroglobulin: carbodiimide molar ratio of 118:1:200. The reaction was carried out for 24 hr at room temperature. The conjugate was then dialyzed against 12 liters of distilled water for 24 hr at 4°. When ^{125}I-GnRH prepared as described below was used to quantitate percent incorporation by determining the percent cpm remaining with the conjugate after dialysis, 20% incorporation was noted. One hundred micrograms of D-[Lys^{6}]-GnRH conjugated to thyroglobulin and emulsified in one ml of complete Freund's adjuvant was injected into two 1.8 kg female New Zealand White rabbits (Hilltop Laboratories). A multiple site subcutaneous injection technique was used.

Iodination. Synthetic GnRH (Bachem, Inc.) or D-[Lys^{6}]-GnRH was monoiodinated by the augmented lactoperoxidase technique as previously described (13). Monoiodinated peptide was then separated from uniodinated peptide by cation exchange chromatography using CM-Sephadex C-25 (Pharmacia Inc., NJ). (16) ^{125}I-GnRH prepared identically also eluted as a single peak on QAE-Sephadex (Pharmacia Inc.) at pH 9.2 (17). Biologically active monoiodinated peptide with a specific activity of 1390 μCi per μg (16) was obtained and stored in elution buffer (0.15 *M* Ammonium Acetate pH 4.5) at 4°.

RIA Protocol. Disposable glass test tubes

0037-9727/78/1584-0643$01.00/0
Copyright © 1978 by the Society for Experimental Biology and Medicine
All rights reserved.

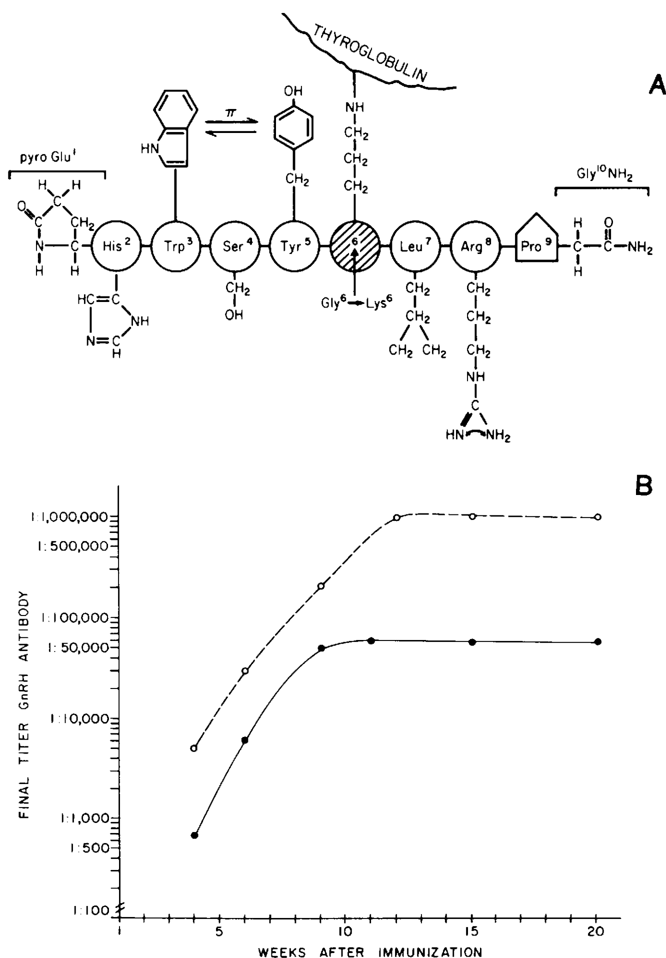

FIG. 1. (A) Primary structure of D-[Lys[6]]-GnRH showing point of conjugation of thyroglobulin. (B) Time course in weeks of obtaining antisera in animal No. 1 (○) and animal No. 2 (●). Final antibody titers binding 20% of added counts are plotted.

(12 by 75 mm) without pretreatment were used. The assay mixture consisted of 600 μl assay buffer (0.05 *M* Phosphate Buffered Saline containing 1% (v/v) normal rabbit serum), 100 μl ^{125}I-GnRH (4000 cpm) in assay buffer, 100 μl of GnRH standard or sample, 100 μl 0.1 *M* EDTA pH 7.4 and 100 μl antibody at various dilutions in assay buffer. The mixture was incubated at 4° for 48 hr. Fifty microliter sheep antirabbit antiserum (R. Henninger, Provo, Utah) was added. Following further incubation at 4° for 24 hr, bound and free hormone were separated by centrifugation for 30 min at 2500 rpm in a Beckman RC-3 centrifuge.

Results. As shown in Fig. 1B antiserum of high titer was obtained in both rabbits by 6 weeks after the initial immunization. The titer increased to 12 weeks at which time 20% of added ^{125}I-GnRH was bound at a final dilution of 1:100,000 in animal no. 1 and 1:1,000,000 in animal No. 2. Nonspecific binding averaged 3% of the added counts. GnRH and D-[Lys[6]]-GnRH reacted equally with the antibodies generated. Unrelated peptides (TRH, arginine vasopressin, LH, FSH, ACTH, prolactin, somatostatin) did not cross react significantly (less than 0.1%). GnRH-COOH (free acid form) did not cross react significantly (0.6%). Des-[pGlu[1]]-GnRH (Beckman, Inc.) cross-reacted 20%. The sensitivity of the assay was between 1 and 3 pg per tube, averaging 1.8 pg in eight assays. The intraassay coefficient of variation was

7%, while interassay variation was approximately 10%. These coefficients were calculated in standard fashion from data obtained on six different assays of five human plasma samples containing varying amounts of added synthetic GnRH.

Following the addition of synthetic GnRH to 100 μl of plasma from normal individuals and serial dilution with plasma from the same individuals, parallel dilution curves were obtained for GnRH diluted in assay buffer alone or in 100 μl plasma in this 1 ml system. In addition, at all dilutions there was complete recovery of added GnRH. GnRH was not detectable in peripheral plasma samples (100 μl) obtained from normal adult male and female rats, rabbits or humans.

When 100 μg synthetic GnRH (Abbott Laboratories) was administered to two normal adult females and three normal males as an intravenous bolus and plasma samples obtained for RIA at −15, 0, 0.5, 1, 1.5, 2, 2.5, 5, 10, 15, 20 and 25 min were assayed, a multiexponential clearance curve with an initial $t_{1/2}$ of 2.5 min was obtained. In addition, after storage at −20° and reassay, samples taken at all these time points diluted in parallel with synthetic GnRH over the range of assay detectability, indicating that cross-reacting but immunochemically different GnRH metabolites did not appear in plasma at measurable concentrations at any time. In addition, 800 μg synthetic GnRH and ^{125}I-GnRH were injected as an intravenous bolus into four mongrel dogs anesthetized with sodium pentobarbital. Plasma samples were obtained at 1, 3, 5, 7, 10, 15, 20, 25, 30, 45, 60, 90, 120, and 150 min. Blood volume was replaced with normal saline. Clearance curves similar to those obtained in the human were observed. Data on one dog receiving both ^{125}I-GnRH and GnRH are shown in Fig. 2. Similar results were obtained on all four animals studied. Plasma samples diluted parallel to synthetic GnRH in the RIA as shown in Fig. 2A. Moreover, the clearance of immunoprecipitable ^{125}I-GnRH was identical to the clearance of immunoassayable GnRH and resolved into the three components shown in Fig. 2B. The initial $t_{1/2}$ of this multiexponential clearance curve was 2.5 min as observed in humans. When half times were calculated from data of separate experiments, the clearance of ^{125}I-GnRH was the same whether administered alone or together with 800 μg synthetic GnRH.

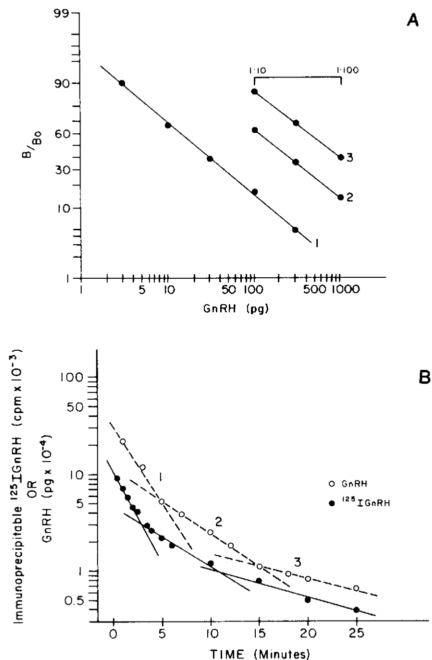

FIG. 2. (A) GnRH RIA standard curve plotted on log-logit coordinates (1) synthetic GnRH; (2) 12 min time point of GnRH metabolism study; (3) 35 min time point of same study. (B) Metabolic clearance of GnRH plotted on log-linear scale. Three components with $t_{1/2}$ of 2.5 min (A), 7 min (B), and 20 min (C) are shown.

Discussion. The use of a biologically superactive synthetic analog of GnRH represents a novel approach to the problem of GnRH RIA development. The plasma GnRH RIA obtained did not significantly improve upon available assays obtained via two-step conjugation or using bifunctional reagents as was originally hoped. However, the simplicity and reliability of the carbodiimide reaction as well as the general availability of D-[Lys^6]-GnRH should encourage the development of this RIA in centers less experienced in peptide RIA design. Significant cross-reaction with des-pGlu^1-GnRH in common with other GnRH RIA's does not in our view diminish the utility of this RIA for studies of GnRH physiology. The best widely used RIA in this regard (Niswender R42), shows 3.8% cross-reaction with des-pGlu^1-GnRH, which is still significant (3). The RIA for D-[Lys^6]-GnRH does not detect endogenous GnRH in 100 μl peripheral plasma, indicating that GnRH cir-

culates at a concentration of les than 10–30 pg/ml. No RIA studies have to date convincingly demonstrated the physiological importance of peripheral plasma GnRH measurements. The data on clearance of GnRH confirm previous work (18–20) by several laboratories, indicating multiexponential clearance with an initial $t_{1/2}$ of 2.5 min in man and experimental animals. Our observation of identical metabolic clearances of ^{125}I-GnRH and GnRH is consistent with uptake by a system not saturated at a peripheral GnRH concentration of 300,000 pg/ml. These data are consistent with our recent observation that membrane preparations from a number of peripheral tissues bind GnRH with an equilibrium association constant of 10^5 l/mole (14, 15).

Summary. Specific, high titer anti-GnRH antisera were obtained following immunization of rabbits with a conjugate of thyroglobulin and D-[Lys^6]-GnRH. D-[Lys^6]-GnRH was chosen to take advantage of an ϵ-amino group in the center of the peptide chain and three carbons away from the chain as a unique point of conjugation. The RIA developed with this antisera was validated for the measurement of GnRH in plasma and hypothalamic supernatants. Studies of GnRH metabolic clearance indicated that multicompartmental clearance by different organs was a probable explanation for the rapid disappearance of GnRH from the peripheral circulation.

1. Matsuo, H., Baba, Y., Nair, R. M. G., and Schally, A. V., Biochem. Biophy. Res. Commun. **43**, 1334 (1971).
2. Geiger, R., Konig, W., Wissman, H., Geisen, K., and Enzmann, F., Biochem. Biophy. Res. Commun. **45**, 767 (1971).
3. Nett, T. M., Akbar, A. M., and Niswender, G. D., Endocrinology **94**, 713 (1974).
4. Kerdelhue, B., Jutisz, M., Gillesen, D., and Studer, D. O., Biochim. Biophys. Acta **297**, 540 (1973).
5. Jonas, H. A., Burger, H. G., Cumming, I. A., Findlay, J. K., and deKretscher, D. M., Endocrinology **96**, 384 (1975).
6. Keye, W. R., Kelch, R. P., Niswender, G. D., and Jaffe, R. B., J. Clin. Endocrinol Metabol. **36**, 1263 (1973).
7. Koch, Y., Wilchek, M., Chobsieng, P., Zor, U., and Lindner, H. R., Biochem. Biophys. Res. Commun. **55**, 616 (1973).
8. Jeffcoate, S. L., Fraser, H. M., Gunn, A., and Holland, D. T., J. Endocrinology **57**, 189 (1973).
9. Arimura, A., Sato, H., Kumasaka, T., Worobec, R. B., Debeljuk, L., Dunn, J., and Schally, A. V., Endocrinology **93**, 1092 (1973).
10. Skowsky, W. R., and Fisher, D. A., J. Lab. Clin. Med. **80**, 134 (1972).
11. Bassiri, R. M., and Utiger, R. D., Endocrinology **90**, 722 (1972).
12. Koch, Y., Baram, T., Hazum, E., and Fridkin, M., Biochem. Biophys. Res. Commun. **61**, 95 (1974).
13. Marshall, J. C., and Odell, W. D., Proc. Soc. Exp. Biol. Med. **149**, 351 (1975).
14. Marshall, J. C., Shakespear, R. A., and Odell, W. D., Clin. Endocrinol. **5**, 671 (1977).
15. Heber, D., Marshall, J. C., and Odell, W. D., Amer. J. Physiol., in press (1978).
16. Heber, D., Odell, W. D., Schedewie, H., and Wolfsen, A. R., Clin. Chem., in press (1978).
17. Reeves, J. J., Tamarsky, G. K., Becker, S. R., Coy, D., Schally, A. V., Endocrinology **101**, 540 (1977).
18. Redding, T. W., Kastin, A. J., Gonzalez-Barcena, D., Coy, D. H., Coy, E. J., Schalch, D. S., and Schally, A. V., J. Clin. Endocrinol. Metabol. **37**, 626 (1973).
19. Jeffcoate, S. L., Greenwood, R. H., and Holland, D. T., J. Endocrinol. **60**, 305 (1974).
20. Virkunnen, P., Lybeck, H., Pantanen, J., Ranta, T., Leppaluoto, J., and Seppala, M., J. Clin. Endocrinol. Metabol. **39**, 702 (1974).

Received January 30, 1978. P.S.E.B.M. 1978, Vol. 158.