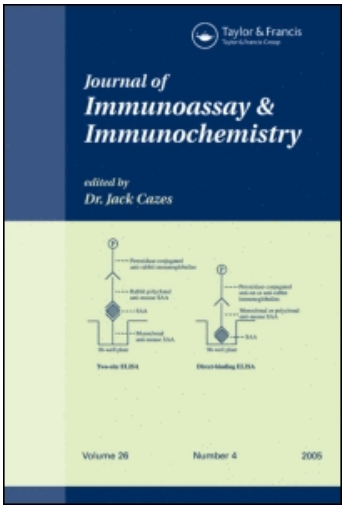

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REANALYSIS OF ANTISERA SPECIFICITIES AND BINDING CHARACTERISTICS OF RAT PITUITARY HORMONE ASSAYS

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ABSTRACT

Rat pituitary hormone radioimmunoassays (RIAs) are widely used in reproductive research, yet data on specificity and binding characteristics of many of the antisera are not widely available. This report characterizes one set of rat antisera supplied by the National Institutes of Health (USA). Rat follicle-stimulating hormone (FSH) and thyrotropin-stimulating hormone (TSH) antisera appear specific, but TSH exhibited significant competition in the rat luteinizing hormone (LH) assay. In addition, statistically significant nonparallelism was demonstrable in all three assay systems. This creates further problems in characterizing antisera cross-reactivity and may make potency estimates for pituitary standards inaccurate. (KEY WORDS: Rat, Pituitary, Radioimmunoassay)

INTRODUCTION

The availability of pituitary hormone antisera has in part been responsible for the significant progress in endocrine re-

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search over the past decade. Several problems encountered in the use of protein hormone antisera, including effects of incubation time, delayed addition of various reagents, and errors in the use of various statistical methods employed for calculating results have recently been clarified (1-3). However, potential problems involved in using antisera of other than absolute specificity have not always been considered. This is due in part to the lack of a commonly accepted definition of assay specificity and the minimal amount of available data on antisera specificity. Recently this has been partially rectified since the National Institutes of Health, USA (NIH) has prepared specificity data for the rat antisera it is currently distributing (4). Data for previous batches of NIH materials, including those still in common laboratory use, are not available, however (5). The purpose of this study was to define the specificities and binding characteristics for one set of antisera distributed by the NIH that has been commonly employed for assay of rat pituitary hormones.

MATERIALS AND METHODS

Reagents

Rat (r) pituitary standards and antisera employed in this study were obtained from the National Hormone and Pituitary Program, NIADDK, NIH. The standards and their biological potencies are shown in Table 1 (data available from NIH). Antisera used, and their final incubation concentrations, were anti-rat LH-S-4 (1:50,000), anti-rat FSH-S-10 (1:12,500), and anti-rat TSH-S-4 (1:25,000).

TABLE 1

Biological Potencies of Rat Pituitary Standards (NIH)[a]

Standard	Biological Activity	Fractional Contamination		
		LH	FSH	TSH
rLH-I-6	1.0 x NIH-LH-S1	---	<0.04 x NIH-FSH-S1	<0.03 USP U/mg
rFSH-I-4	150 x NIH-FSH-S1	<0.002 x NIH-LH-S1	---	---
rTSH-I-4	35 IU/mg	0.02 x NIH-LH-S1	<0.1 x NIH-FSH-S1	---
rPRL-I-1	30 IU/mg	none	none	<0.1 USP U/mg
rLH-RP-1	0.03 x NIH-LH-S1	---	0.54 x NIH-FSH-S1	0.22 USP U/mg
rFSH-RP-1	2.1 x NIH-FSH-S1	0.02 x NIH-LH-S1	---	0.3 USP U/mg
rTSH-RP-1	0.22 USP U/mg	0.03 x NIH-LH-S1	0.54 x NIH-FSH-S1	---
rPRL-RP-1	11 IU/mg	none	none	none

[a] Data supplied by NIH at time of receipt of standards.

Sodium ^{125}I for hormone iodination was obtained from Amersham Nuclear Corp. (Arlington Heights, IL) at a specific activity of 15-17 mCi/μg. Chloramine-T for oxidation was obtained from Eastman Kodak Co. (Rochester, NY). Goat anti-rabbit gamma globulin for precipitation was obtained from Antibodies, Inc. (Davis, CA). Normal rabbit serum was prepared in this lab.

Phosphate-buffered saline (PBS) contained 0.01 M sodium phosphate, 0.15 M sodium chloride, and 0.15 M sodium azide, pH 7.6. The assay buffer for RIA consisted of 10 mg/ml bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS. Antisera were diluted with a solution of 3% (vol/vol) normal rabbit serum in PBS containing 0.05 M sodium EDTA (Fisher Scientific, Fair Lawn, NJ).

Preparation of [^{125}I]iodo Hormones

Purified rat assay standards (2 μg protein) were labeled with 2 mCi ^{125}I by reaction in the presence of 15 μg Chloramine-T and 25 μl 0.5 M sodium phosphate, pH 7.6. Iodination was to a mean specific activity of 120-150 μCi/μg. Labeled hormones were purified by gel filtration on a Sephadex G-75 (Sigma) column. This procedure was carried out at 22°C on a 1.0 x 12 cm column pre-equilibrated with 20 mg/ml BSA in PBS. Fractions of 0.25 ml were collected and aliquots were counted to obtain an elution profile. The iodinated hormone was diluted in 10 mg/ml BSA in PBS to 20,000 cpm (~0.05 ng) per 0.1 ml solution.

RIA of Rat Hormones

Procedures for RIA were those recommended by NIADDK for use with the supplied materials. Total initial assay volume was 0.8

ml; each assay tube contained 200 µl of antibody, 100 µl of [^{125}I]iodo hormone, and 500 µl of unlabeled hormone in PBS. Initial incubation was for 72 h at 22°C, with a second incubation of 24 h following the addition of 200 µl of the precipitating antibody. Samples were centrifuged at 1000 x g for 30 min. Precipitates were counted in a gamma spectrometer (Prias PGD, Packard Instruments, Downers Grove, IL); counting efficiency was 70-75%. Most assays were independently replicated in two laboratories under identical conditions using similar instrumentation. Results within each assay were plotted as amount bound (percentage of maximal binding, B/B_0 x 100) versus log concentration of standard added. The data presented (Fig. 1 and Table 2) is from one laboratory (NUMS) only. Results in both laboratories were similar.

Statistical Analysis

The slopes of the competition curves were estimated using all data points in the linear portion of the curve; that is, all data points whose mean of three replicates fell within the range of $0.20 \leq B/B_0 \leq 0.80$. Analysis of covariance (6) was used to analyze the different hormone standards for each antiserum. For all three systems the analysis of covariance (ANCOVA) rejected the null-hypothesis of equal slopes ($p \leq 0.025$, Table 2). Because of the nonparallelism, relative potency varies with dose. Point estimates of relative potency and 95% individual confidence intervals (using Fieller's theorem) were obtained for the base 10 logarithm of standard added that corresponded to a 50% inhibition level for each hormone standard (7).

RESULTS

rLH Assay System

Results of the rLH assays are shown in Fig. 1a and Table 2. rLH-1-6 competed 5 times more strongly than did rLH-RP-1. rFSH-1-4 did not interfere with the LH assay appreciably, but rFSH-RP-1 had a cross-reaction of 6.8% compared to rLH-I-6, reflecting its probable contamination with LH (Table 1). No decrease in binding was demonstrated in the presence of rPRL, even when the concentration of rPRL exceeded the concentration of labeled LH by a factor of 10^5. The most significant cross-reaction found was that for the two TSH standards. Cross-reaction between rLH-I-6 and rTSH-RP-1 was 17.6%, while that between rLH-I-6 and rTSH-I-4 was 21.9%.

Significant nonparallelism was demonstrated ($p \leq 0.025$) between the competition curves by ANCOVA (Table 2).

rFSH Assay System

Results of the homologous rFSH assay system are shown in Fig. 1b. When compared to the rFSH-I-4 standard, no significant cross-reactions (>1.0%) were noted for any of the preparations tested except for rFSH-RP-1 (3.9%). No depletion of [^{125}I]iodo FSH could be demonstrated for rLH-I-6 or rPRL-I-1 up to levels of 5×10^5 ng of standard.

rTSH Assay System

As in the rLH and rFSH systems, nonparallelism was observed ($p < 0.001$) for competition curves in the rTSH assay system (Fig. 1c). Of the three iodination standards, only rFSH-I-4 had any degree of cross-reaction as compared to rTSH-I-4 (Table 2). All

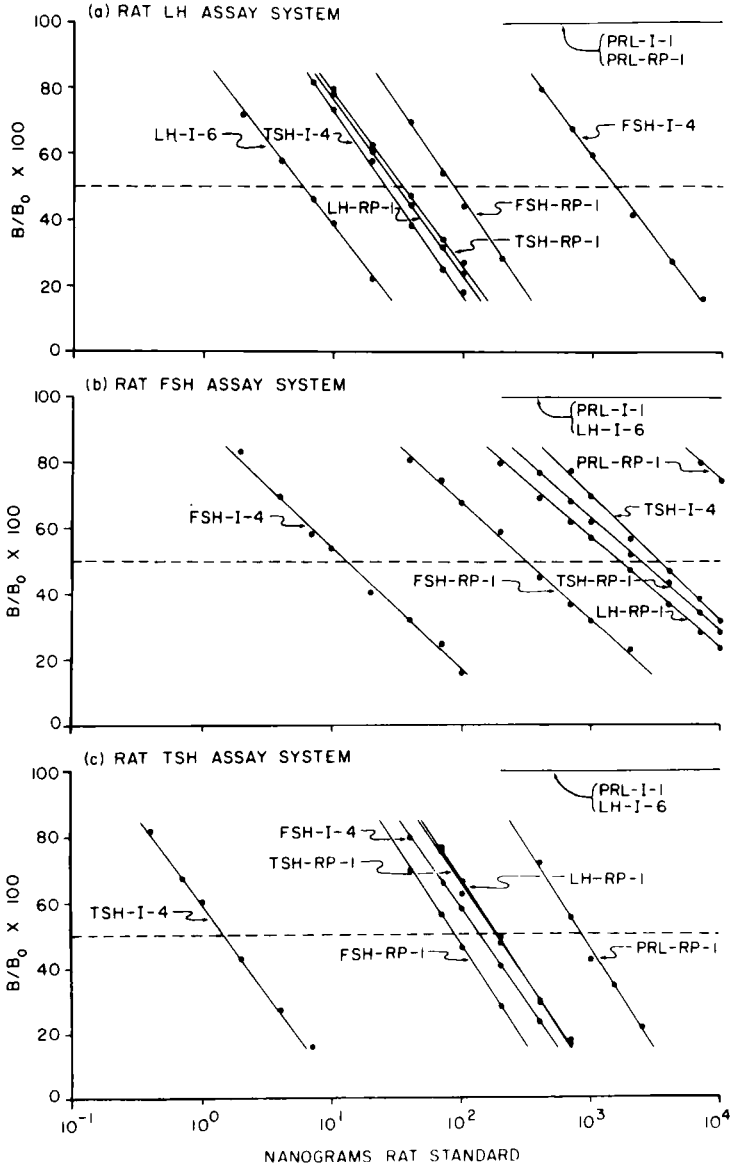

Figure 1. Competition curves of various hormone standards in three homologous rat assay systems. Each plotted point represents a mean of 3 determinations; calculations were done using individual determinations. Assays were performed as described in the text. Slopes and correlation coefficients are detailed in Table 2.

TABLE 2

Statistical Analysis of Rat Assay Systems

Assay System	P-value[a] (ANCOVA)	Standard	Sample Size	Slope	Coefficient of Correlation	50% Response Level and 95% Confidence Intervals[b]			Relative Potency at 50% Response[c]
rLH	0.0251	LH-I-6	15	-0.5052	-0.9904	0.6450	0.7556	0.8655	1.0000
		LH-RP-1	15	-0.5381	-0.9990	1.4741	1.5114	1.5486	0.1755
		TSH-I-4	12	-0.5877	-0.9942	1.3321	1.4158	1.4991	0.2187
		TSH-RP-1	15	-0.5314	-0.9877	1.4220	1.5552	1.6885	0.1586
		FSH-I-4	15	-0.5365	-0.9940	3.0811	3.1700	3.2596	0.0039
		FSH-RP-1	12	-0.5971	-0.9921	1.8433	1.9216	1.9996	0.0682
rFSH	0.0044	FSH-I-4	18	-0.3635	-0.9032	0.9516	1.1092	1.2655	1.0000
		FSH-RP-1	21	-0.3644	-0.9944	2.3973	2.5133	2.6292	0.0394
		LH-RP-1	24	-0.3350	-0.9957	3.0881	3.2017	3.3154	0.0081
		TSH-I-4	21	-0.3781	-0.9941	3.4131	3.5323	3.6513	0.0038
		TSH-RP-1	21	-0.3416	-0.9965	3.2909	3.3800	3.4699	0.0054
		PRL-RP-1	6	-0.3572	-0.9674	4.4899	4.6859	5.0621	0.0003
rTSH	0.0002	TSH-I-4	12	-0.5406	-0.9977	0.1256	0.1733	0.2210	1.0000
		TSH-RP-1	12	-0.5888	-0.9934	2.1654	2.2468	2.3289	0.0084
		FSH-I-4	15	-0.5667	-0.9967	2.0630	2.1291	2.1954	0.0111
		FSH-RP-1	12	-0.5940	-0.9963	1.8817	1.9347	1.9876	0.0173
		LH-RP-1	12	-0.6051	-0.9938	2.1971	2.2757	2.3553	0.0079
		PRL-RP-1	15	-0.4966	-0.9887	2.8777	3.0010	3.1233	0.0015

[a] P-value for test of the null-hypothesis of equal slopes, from analysis of covariance.
[b] Dose (ng) estimate and 95% confidence intervals for observation at 50% response, log-10 scale.
[c] Amount of homologous iodination standard (ng) required to decrease binding of the [^{125}I]iodo hormone by 50% relative to the amount of competing standard (ng) required to do the same.

four RP-1 standards competed to a minor extent, but this antiserum was the most specific of the three examined.

DISCUSSION

Although various RIA standards and antisera are available for rat pituitary hormones, use of the kits or materials available from the NIH (NIADDK) have been reported on most frequently and have gained the widest acceptance among investigators in the United States. Such uniformity leads to data which is easily interpretable and repeatable, enabling the integration of data worldwide. However, it is clear that any cross-reactivity by the antiserum among pituitary hormones can lead to errors in reported data and conclusions which may not be warranted.

In the present study we measured the cross-reactivities of one set of NIH materials, using homologous rat LH, FSH, and TSH assay systems. It is apparent from Table 1 that the iodination standards are the most highly purified hormones available from the NIH. Solano, Dufau, and Catt (8) found a 3-fold difference in the ratio of biologic and immunologic activity between the 2 NIH standards for rLH, indicating that the difference was mainly due to the presence of biologically inactive LH in the RP-1 standard. We have therefore defined cross-reactivity with respect to the more purified iodination standards rather than to the RP-1 standards, where cross-reaction in some cases would exceed 100%.

The data presented in this study indicate no potential problems due to cross-reactivity in either the rat FSH or TSH RIAs. Each of these antisera is specific with respect to the purest pre-

parations of the homologous hormone. Of potential concern, however, is the degree of cross-reactivity found in the rLH assay system with reference to TSH. The specificity of this antiserum for rLH appears to be quite low, and problems may arise when attempting to define the concentrations of serum LH in hypothyroid animals or in any condition where serum levels of TSH are elevated (9,10). Compared to the iodination standard a 22% cross-reaction was found with TSH. Therefore, if equal concentrations of LH and TSH are present in the serum of a rat, 22% of the LH measured may be accounted for by TSH, giving an estimate for LH that is significantly in error. Since basal serum levels of TSH in the rat are estimated at 2-10 times the tonic levels of LH (9,10), the cross-reactivity must be acknowledged and adequately accounted for when interpreting the results of gonadotropin assays.

The degree of nonparallelism exhibited in all three assay systems, while not a serious problem in the everyday use of these assays, does raise the question of reliable potency estimates. This statistical lack of parallelism is reproducible between assays, and probably indicates nonidentical antigenic determinants (11,12). Since the α-subunits of these glycoprotein hormones are assumed to be identical within given species (13), the nonparallelism may be representative of conformational changes induced in the antigenic α-subunit as it combines with the various β-subunits to produce the native hormones. Recent experiments by Strickland and Puett (14) have shown that a common ovine α-subunit, when artificially combined with various β-subunits, produces nonparallel dilution curves. Nonparallelism is also seen in

recent data provided by NIH using native rat glycoprotein hormones. This apparently has not been recognized as such when computing relative potency, however. In this study relative potency was defined only for a single point (ie: the 50% response level), since estimates prepared from nonparallel competition curves may be misleading and inaccurate.

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