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Assays of Antibodies to a C-Terminal Peptide or the Entire β-Subunit of Human Chorionic Gonadotropin

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Antibodies were assayed in serum samples obtained from rabbits or women immunized with a vaccine based on a C-terminal peptide (109–145; CTP) of the β-subunit of human chorionic gonadotrophin (hCG) with use of a ligand-binding assay. In rabbit samples, two types of assay were used. The first "homologous" type was based on CTP as tracer and standard. In the second "heterologous" type, directly reflecting the hCG-neutralizing potency, hCG was used as tracer and standard. The equilibrium constants of antibodies were substantially higher in the homologous than in the heterologous assay, indicating that the fit of hCG to the antibodies was worse than that of CTP. This was further confirmed by very low cross-reaction values of hCG. In addition, hooks occurred in Scatchard plots when the heterologous assay type was used, both with rabbit and human samples. However, a high correlation between the results of the homologous and heterologous assay was observed ($r = 0.97$; $P < 0.05$). Therefore, it is envisaged that the possibility of using the homologous, analytically less complex assay will be further investigated in future clinical studies. Antibodies raised in women to the β-subunit of hCG had equilibrium constants higher by one to two orders of magnitude than those of the anti-CTP antibodies. The present definition of a threshold pregnancy-preventing level of antibodies disregards their avidity. It is suggested that in future studies, the problem of varying avidity could be solved by individually adjusting the threshold levels with respect to antibody avidity. J. Clin. Lab. Anal. 12:60–64, 1998.

Key words: pregnancy; immunization; quantitation; threshold; contraception

INTRODUCTION

Human chorionic gonadotrophin (hCG) is a very important embryonic signal for the initiation of implantation and maintenance of early pregnancy. The expression of genes for hCG has been shown in a preimplantation embryo (1).

To block the early action of hCG has become a target of a projected novel method for family planning. The aim is to inactivate hCG through binding it to circulating antibodies that had been raised by immunization in women desiring contraception.

Either intact β-subunit of hCG (2), or carboxy terminal peptide (109–145; CTP) of the β-subunit (3,4) have been used as basic constituents of the vaccine. The former vaccine has already undergone clinical efficacy tests (2), the latter has been subjected to clinical safety testing (Phase I study; ref. 4), and efficacy testing is being planned.

The immunization method using CTP in the vaccine has been given preference by the WHO Special Programme for Research, Development, and Research Training in Human Reproduction because the antibodies produced by this vaccine do not cross-react with hormones that are structurally close to hCG, for example luteinizing hormone (4).

It is obvious that the quantitative measurement of the antibodies is of paramount importance in the development of the optimum immunization regime and later on in its practical clinical application, as it has to be decided individually for each woman whether or not her antibody levels have reached a protective threshold level.

The aim of the present work is to elucidate some problems associated with quantitation of the hCG binding to circulating anti-CTP antibodies.

MATERIALS AND METHODS

Standards

Synthetic carboxy terminal peptide (CTP) representing aminoacid sequence 109–145 of the C-terminal region (mol wgt. 3,872) of the β-subunit of human chorionic gonadotrophin (hCG), hCG standard (CR127, Center for Population

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Research, National Institutes of Health, Bethesda, MD; expressed in mass units, assumed mol. weight = 38,000), and CTP with tyrosine covalently bound in 109-position (CTP-tyr; see ref. 5; mol. wgt. 4,035) were generously donated by Dr. V. Stevens.

Iodination

Iodination of hCG or CTP-tyr was carried out by oxidation with Chloramine T. The compound to be iodinated (10 μg) in phosphate buffer (30 μL; 0.5 mol/L; pH 7.5) was mixed with potassium ^{125}iodide (1 mCi in 10 μL) and Chloramin T (10 μg/10 μL). After 30 sec of continuous mixing, the oxidation reaction was stopped by sodium bisulfite (20 μg/20 μL). After adding potassium iodide (150 μg/30 μL), phosphate buffer (200 μL; 0.05 mol/L) containing 0.5% (w/v) of bovine serum albumin (BSA) was added and the solution was subjected to chromatography on a Sephadex column (G50 for hCG and G25 for CTP-tyr) using the BSA buffer as eluent. The peak of the iodinated material was used as tracer.

Serum Samples

Human samples were kindly donated by Drs. Jones and Talwar, the former being obtained after immunization with CTP (4), the latter with hCG β-subunit (2). Rabbit samples containing anti-CTP antibodies were donated by Dr. Stevens.

Ligand-binding Assay

Each sample (human or rabbit) was brought to an appropriate dilution with phosphate buffer (10 mmol/L, pH 7.5, containing 50 mmol/L of EDTA and 15% calf serum). The diluted sample (50 μL) was incubated with standard (zero and eight doses increasing from 0.0457 to 10 ng in multiples of 3) and tracer (cpm equivalent to 0.1 ng), each in 50 μL of phosphate buffer containing 1% BSA, for 48 h at 4°C. Thereafter, phosphate buffer (50 μL) containing 50 mmol/L of EDTA and 40% calf serum was added to each tube and the bound fraction was precipitated by 200 μL of phosphate buffer containing 25% polyethylene glycol (average mol. wgt. 8,000). After centrifugation, radioactivity was measured in the precipitate.

For each sample a Scatchard plot (6) was constructed and the concentration of binding sites were computed by means of LIGAND program (7). In spite of the apparent presence of two families of binding sites (Fig. 1A), the straight line computed for a single binding site was used for the quantitation. In model calculations, a single binding site was assumed.

The molar concentrations found in the heterologous assays are to be considered approximate as their calculation was based on a theoretical molecular weight of 38,000 for the hCG preparation CR127. Although the purity of this preparation is assumed to be high, it is not absolute, and, consequently, the real concentrations are somewhat lower than those reported here.

RESULTS

Rabbit Samples

A "homologous" ligand-binding assay of anti-CTP antibodies was based on CTP-tyr used as both tracer and standard. Scatchard plots of regular patterns, characteristic for two families of binding sites, were obtained (see Fig. 1A for an example). The concentrations and equilibrium constants (K) resulting from assays of rabbit serum samples are shown in Table 1.

Cross-reactions of hCG with anti-CTP antibodies were very small (Table 1; Fig. 2). Large amounts of hCG were needed to compete with CTP-tyr tracer for the antibodies.

"Heterologous" assays of anti-CTP antibodies using hCG as both tracer and standard resulted in concentrations and equilibrium constants that were substantially lower than those in the homologous assay (Table 1). In addition, the Scatchard plots were irregular by exhibiting a hook in the first part of the Scatchard plot (Fig. 1B). A correlation coefficient of 0.957 ($P<0.05$) was found between the concentrations of the homologous and heterologous assay.

Human Samples

The concentrations and equilibrium constants of human samples originating from immunization with the hCG β-subunit and CTP vaccine, respectively, are shown in Table 2. The equilibrium constants of antibodies resulting from the latter immunization were lower by one to two orders of magnitude. The hooked shape of Scatchard plot was similar to that seen in the rabbit samples.

DISCUSSION

There has been an extensive discussion on the mode of action of the anti-CTP antibodies (8–11). The inhibition of hCG binding to its receptor has been suggested (8,9) to be the main mechanism of action of anti-hCG and anti-CTP antibodies. Another viable concept is the interception of hCG in circulating blood by the antibodies before it can reach the receptors. This other concept makes it very important to measure concentrations of circulating antibody levels in clinical immunization studies.

It would seem natural to use the "homologous" approach (CTP as both tracer and standard) for the assays of antibodies elicited by immunization with CTP. Such assay, however, do not provide direct information on the binding of hCG, which is the main objective. Therefore, "heterologous" assays of anti-CTP antibodies using hCG as both tracer and standard have been used in spite of the fact that the concentrations and equilibrium constants are substantially lower than in the homologous assay variant, apparently due to a worse fit of hCG molecules into the binding sites of the anti-CTP antibody. In addition, the Scatchard plots are usually irregular in the heterologous assays by exhibiting a hook in the first part of the

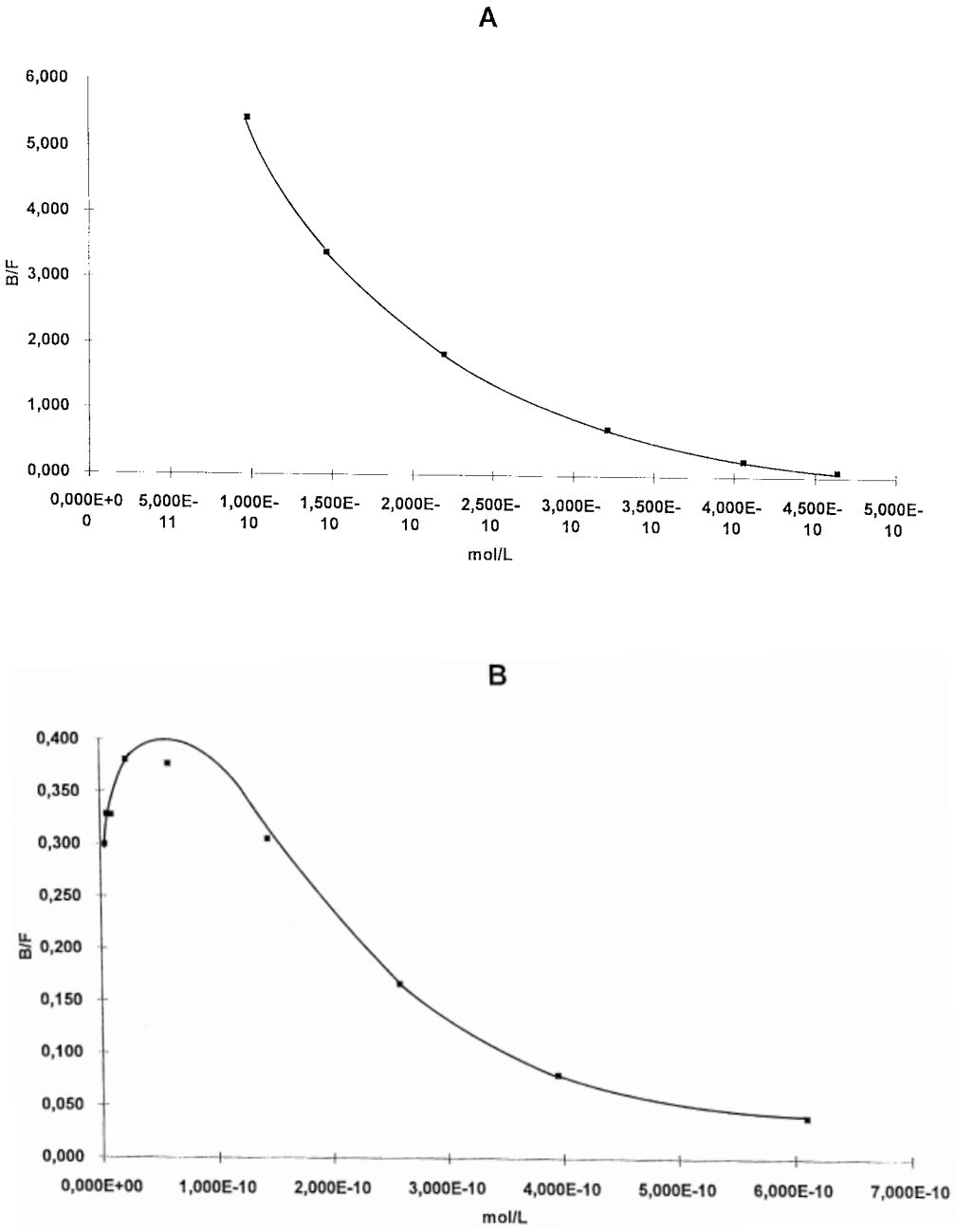

Fig. 1. Examples of Scatchard plots in anti-CTP antibody assays. **A**. Homologous assay (CTP-tyr as tracer and standard). **B**. Heterologous assay (hCG as tracer and standard).

plots that may be due to the formation of circular complexes of hCG with the antibodies (12). Moreover, the results of the heterologous assays are not fully accurate due to the uncertainty about the degree of purity of the hCG standard.

In spite of all these problems, the heterologous assays should be able to safely detect high levels of anti-CTP antibodies. Prudence should be used, however, when interpreting results indicating low levels, especially those in the neighborhood of the threshold level for the prevention of pregnancy. As a high correlation may generally exist between the homologous and heterologous assays, it may be envisaged that the homologous assays, analytically less ambiguous, will be included in future clinical studies, and the correlation of results will be further investigated in a larger number of samples to see whether or not it would be feasible to use the homologous assay only.

TABLE 1. Concentrations and Equilibrium Constants (K) in Two Types of Ligand-binding Assay of Antibodies Raised in Rabbits to CTP[a]

	Homologous assay (CTP-tyr tracer + CTP-tyr standard)		Heterologous assay (hCG tracer +hCG standard)		Cross-reaction (CTP-tyr tracer +hCG standard)
Rabbit serum[b] no.	Concentration[c] (nmol/L)	K (L/mol) $\times 10^9$	Concentration[c] (nmol/L)	K (L/mol) $\times 10^9$	%[d]
12	256	12.1	131	1.4	0.4
14	1060	3.6	347	0.6	5.1
16	1180	3.9	325	0.9	2.8
28	627	8.8	202	1.9	2.9
LCP-795	1970	5.3	840	1.2	0.2 (by extension)

[a]Same serum dilution (1:200) was used in both types of assay.
[b]Samples were kindly donated by Dr. V. Stevens.
[c]The calculation of correlation between the results of the two types of assay resulted in r = 0.957 (n = 5; $P<0.05$).
[d]Measured at 50% binding in the plots of logit of bound/total vs. log of dose because the cross-reaction lines were not parallel with the "standard" lines (see Fig. 2).

Another problem associated with the quantitative analysis of anti-CTP antibodies is that of threshold levels. The concentrations of antibodies exceeding the threshold level are expected to provide fertile women with protection against pregnancy by neutralizing hCG produced by the early embryo. On the basis of clinical results, such a level was suggested to be 50 ng/mL (13) corresponding to ~1.5 nmol/L. This level has been derived for the antibodies raised to the β-subunit of hCG. These antibodies have, according to our measurements, equilibrium constants of the order of magnitude of 1×10^{10} L/mol. Would it be justified to apply the same threshold level for the anti-CTP antibodies with equilibrium constants lower by one or two orders of magnitude, i.e., 1×10^9 to 1×10^8 L/mol?

According to the law of mass action, a small amount of free hCG (not bound to antibody) always must be present, its concentration being dependent upon the equilibrium constant—the avidity of the antibody. The law of mass action is described by the following equation:

$$K = [Ab{:}hCG]/[hCG][Ab] \qquad \text{(eq. 1)}$$

where K (L/mol) = equilibrium constant,
[Ab:hCG] (mol/L) = complex of antibody with hCG,
[hCG] (mol/L) = free (not bound to antibody) hCG,
[Ab] (mol/L) = free antibody.

Concentrations of the complex and free fractions can be calculated when the total concentrations of antibody and hCG are known:

Total concentration of the antibody ([Ab:hCG] + [Ab]) = a
Total concentration of hCG ([Ab:hCG] + [hCG]) = h

$$[Ab{:}hCG] = \{(aK+hK+1) - \sqrt{[(aK+hK+1)^2 - 4K^2ah]}\}/2K$$
$$[Ab] = a - [Ab{:}hCG]$$
$$[hCG] = h - [Ab{:}hCG] \qquad \text{(eq. 2)}$$

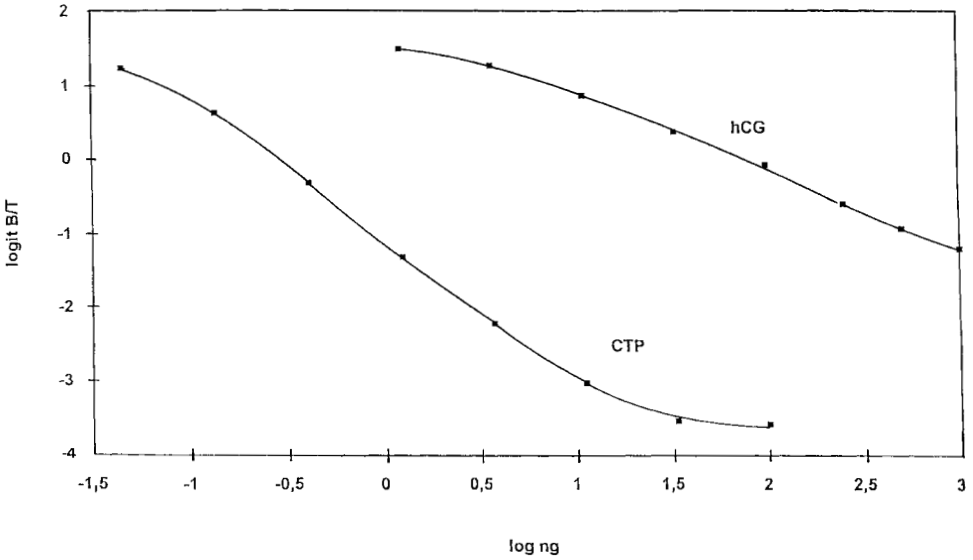

Fig. 2. Cross-reaction of hCG with anti-CTP antibodies in rabbit sample No. 12. The CTP line was obtained with CTP-tyr as tracer and standard, the hCG line with CTP-tyr as tracer and hCG as standard. Logit = 0 corresponds to 50% binding.

TABLE 2. Concentrations and Equilibrium Constants of Antibodies in Human Serum Samples Obtained After Immunization With β-hCG or CTP Vaccine

Sample no.	nmol/L	K (L/mol) $\times 10^9$
	β-hCG vaccine[a]	
1	0.9	16.0
2	0.8	16.1
3	1.9	19.9
4	1.6	23.5
5	18.1	14.2
6	11.2	18.9
7	8.7	25.4
8	8.7	33.2
9	37.9	25.2
10	97.1	11.2
11	97.1	11.3
12	96.6	18.6
	CTP vaccine[b]	
2	6.3	0.53
6	8.1	0.95
7	2.0	0.64
8	20.6	0.33
9	3.1	0.48
11	2.5	0.46

[a]Samples were obtained from Dr. Talwar. hCG was used as both tracer and standard in the assay.
[b]Samples were withdrawn in the Phase I study (4). The heterologous type of assay was used, i.e., hCG was used as both tracer and standard.

The derivation of equation (2) can be obtained from the authors upon request.

As the purpose of the immunization is to inactivate hCG by its binding to antibodies, it must be assumed that the free portion is the one that is biologically active and that, if increased above a certain level, supports pregnancy.

Consequences of the difference in the avidities of the two antibody types for the concentration of the free hCG fraction may be elucidated by a model calculation. For such a calculation, the following arbitrary assumptions have been made: (1) the equilibrium constants are 1×10^{10} L/mol and 1×10^{8} L/mol for the anti-hCG and anti-CTP antibodies, respectively, (2) the total concentration of each antibody is equal to the threshold level of 1.5 nmol/L, and (3) the total hCG concentration is as low as 1.5 pmol/L in both cases. As a result of the calculation, the concentration of free hCG is 0.094 and 1.30 pmol/L for $K = 1\times10^{10}$ L/mol and $K = 1\times10^{8}$ L/mol, respectively. Thus the difference in equilibrium constants of two orders of magnitude causes a relatively large difference between the concentrations of the free hCG fraction. It is to be added that the above concentration of total hCG may be the sum of the endogenous hCG (14) and that produced by a very early embryo.

The above calculations suggest that the threshold levels should be adjusted in each immunized individual with respect to the avidity of antibodies produced. To give an example of two extreme hypothetical cases: if 1.5 nmol/L is the threshold level for an individual with antibodies having $K = 1\times10^{10}$ L/mol, then, 150 nmol/L should be the threshold level for another individual with antibodies of $K = 1\times10^{8}$ L/mol, if the same concentration of free hCG should be present in both cases.

It would seem of great interest to investigate further the usefulness of the individual adjustments of threshold levels. Such studies appear to be important in view of the already seen and expected between-subject fluctuation in the avidity of antibodies.

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