Preliminary Studies on the Indium Slide Immunoassay for Estimation of Human Chorionic Gonadotropin and Antihuman Chorionic Gonadotropin Antibody (43170)

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Abstract. **Human chorionic gonadotropin (hCG) is synthesized and secreted as early as 170 hr after fertilization and has been used as an index for pregnancy. Neutralization of hCG with a β-subunit hCG vaccine(s) has been proposed as a contraceptive technique. To monitor the duration of effectiveness of the vaccine, it will be necessary to monitor the anti-hCG antibodies, especially those responsible for inhibiting the hCG bioactivity. We report a simple, rapid technique using an indium slide immunoassay for the qualitative estimation of hCG and to monitor a bioeffective anti-hCG antibody. The sensitivity of the indium slide assay to measure hCG ranged from 1 μg/ml to 1 ng/ml, depending on the format of the assay. The indium slide assay also detected anti-hCG antibodies generated against a specific determinant on hCG recognized by a neutralizing monoclonal antibody (P_3W_{80}) in women immunized with a contraceptive vaccine.**

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Human chorionic gonadotropin (hCG) is synthesized and secreted as early as 170 hr after fertilization (1) and has traditionally been used as an index for pregnancy. In a continuing quest for simple and sensitive immunoassays, a variety of enzyme immunoassays (2–4), erythroimmunoassays (5), and dot enzyme immunoassays (6) have been developed, some of which have attained sensitivity approximating that of radioimmunoassays. A visual method to detect antigen or antibody employing indium-coated slides has been reported with clinical samples (7, 8).

Immunoneutralization of hCG has been proposed as a contraceptive measure (9). This can be achieved by immunizing women with vaccines consisting of the β-subunit of hCG (β-hCG) or of β-hCG annealed to the α-subunit of ovine luteinizing hormone (α-oLH) and linked to carrier proteins such as tetanus toxoid (TT) or diphtheria toxoid (DT). To monitor the duration of effectiveness of the vaccine, it will be imperative to follow anti-hCG antibodies. For this purpose a simple and rapid method is required. Furthermore, it will be more meaningful to measure the particular antibodies responsible for the neutralization of hCG bioactivity instead of the total antibody population. We report the feasibility of the indium-slide immunoassay for the qualitative estimation of hCG and to monitor a bioeffective anti-hCG antibody.

Materials and Methods

Indium-Coated Slides. Indium-coated plastic sheets were obtained from Optical Coating Laboratories, Inc., Santa Rosa, CA. The sheets were cut into strips approximately 5 mm in width and 25 mm in length, to fit into 12- × 75-mm test tubes.

Antigen. Highly purified hCG (10,000 IU/mg) calibrated against the Second International Standard for hCG was provided by Mr. P. Shai of the National Institute of Immunology, New Delhi, India.

Serum. Serum samples from women immunized with β-hCG or β-hCG-α-oLH linked to TT or DT (Phase I clinical trial in India) were obtained. Four pools of sera from five women each were evaluated for

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the assay. Pool 1 consisted of preimmunized serum samples, Pool 2 of serum samples having a low anti-hCG antibody titer (30 ng of hCG binding/ml), Pool 3 having a moderate titer (100 ng of hCG binding/ml), and Pool 4 having a high antibody titer (300 ng of hCG binding/ml) as determined by radioimmunoassay.

Capture Antibody. Monoclonal antibody P_3W_{80} specific for hCG (10) was used for coating the indium slide. It recognizes a conformation common to the native hormone and the β-subunit of hCG, has low reactivity (less than 1%) with the α-subunit of hCG or human luteinizing hormone (LER-960), and no reactivity with follicle-stimulating hormone and thyroid-stimulating hormone. Its association constant for binding with hCG was $K_a = 3.0 \times 10^{10}$ liters/mol (11). It neutralizes the hCG bioactivity both *in vitro* and *in vivo* (12). Immunoglobulins were isolated from ascites by precipitation with ammonium sulfate at 40% saturation, followed by dialysis in Tris-buffered saline (TBS), pH 7.4 (0.1 *M* Tris, 0.05 *M* NaCl).

Additional Antibodies. Monoclonal antibody P_22_3 conjugated to horseradish peroxidase (HRP) was used for the enzyme enhancement of the assay. It recognizes α-hCG, hCG, and pituitary gonadotropins, i.e., human luteinizing hormone and human follicle-stimulating hormone but not the β-hCG (13). Goat antiserum to highly purified β-hCG produced at the National Institute of Immunology by conventional immunization was used as polyclonal antibody for enhancement of the assay.

Preparation of the Enzyme-Antibody Conjugate. Five milligrams of monoclonal anti-a-hCG antibody (P_22_3) were coupled to 10 mg of HRP by the one-step glutaraldehyde procedure (14). The conjugate was centrifuged to remove any precipitates, dialyzed against phosphate-buffered saline (pH 7.4; 0.01 *M* phosphate, 0.15 *M* NaCl) overnight at 4°C, mixed with an equal volume of bidistilled glycerol, and stored at −20°C.

Immunoassay. The assay is based on two principles. The first is that no more than a monolayer of protein will adhere to a glass or plastic surface. Subsequent protein binding takes place due to specific antigen-antibody reaction. The second principle is that adsorbed proteins on the indium-coated surface turn the light brown surface to a darker brown—the more protein, the darker the color. Thus, a single layer is distinguishable from multiple layers of proteins (by the amount of light transmitted through slide) (7). This phenomenon is presumably due to the proteins creating a dielectric layer, which increases scattering of light. The reaction can be read with the unaided eye.

Immunoassay of hCG. The assay, a sandwich method, was carried out as described by Burek *et al.* (15) with the following modifications. Five microliters of anti-B-hCG monoclonal antibody (P_3W_{80}) diluted in 0.9% saline at concentrations of 50, 25, and 12.5 μg of antibody/ml were applied to each indium slide and incubated for 30 min in a humidified chamber at room temperature, creating visible spots. After incubation, the indium slide was rinsed with distilled water and further incubated in 2% normal goat serum (diluted in 0.9% saline) for 30 min to mask the capture-antibody spot. The slides were then incubated with the agitation in test tubes containing different amounts of hCG for 60 min at room temperature. Visible spots reappeared after specific binding of hCG to the antibody. The hCG bound to the solid surface was amplified by one of the following procedures: (i) incubation for 30 min with monoclonal anti-a-hCG antibody (50 μg of antibody/ml) diluted in TBS; (ii) incubation for 30 min with polyclonal goat anti-hCG antibody (50 μg of antibody/ml) diluted in TBS; or (iii) incubation for 30 min with monoclonal anti-a-hCG antibody-HRP conjugate (10 μg of antibody/ml) diluted in TBS followed by washing with distilled water and subsequent development of precipitable color by incubating with 3′,3′-diaminobenzidine (DAB; 1 mg/ml of TBS containing 0.03% H_2O_2) for 10 min.

Immunoassay of anti-hCG antibody. This is a competitive assay. To monitor bioeffective anti-hCG antibody titers, P_3W_{80}-coated indium slides were incubated for 60 min with 50 ng of hCG/ml mixed with a 1/20 dilution (final) or sera obtained from immunized women. If specific antibodies were present, a reduction in the intensity of the spot on the slide should be seen. Slides were washed and further incubated for 30 min with monoclonal anti-hCG antibody-HRP conjugate and processed as described above.

Results

Human Chorionic Gonadotropin. If the slides were incubated only with hCG, the limit of detection for hCG was about 1 μg of hCG/ml (Fig. 1). When the sandwich was completed with monoclonal anti-α-hCG antibody, the sensitivity was extended to approximately 500 ng of hCG/ml. The technique could detect as little as 100 ng of hCG/ml when amplified with the polyclonal goat anti-hCG antibody. To improve the sensitivity even further, monoclonal anti-α-hCG antibody-HRP conjugate was used, and indium slides were then developed in DAB. Using this system, as little as 1 ng of hCG/ml could be detected reproducibly (Fig. 1).

Estimation of Anti-hCG Antibody in Women Immunized with B-hCG-a-oLH Linked to TT or DT. The feasibility of measuring anti-hCG antibodies generated against the determinant recognized by P_3W_{80} (known to neutralize hCG bioactivity) in serum of immunized women was explored. As a control, indium slides bearing P_3W_{80} and incubated with 50 ng of hCG and a 1/20 dilution of preimmunized sera showed dark spots comparable to the one incubated with 50 ng of hCG alone. This indicates no anti-hCG antibody and also

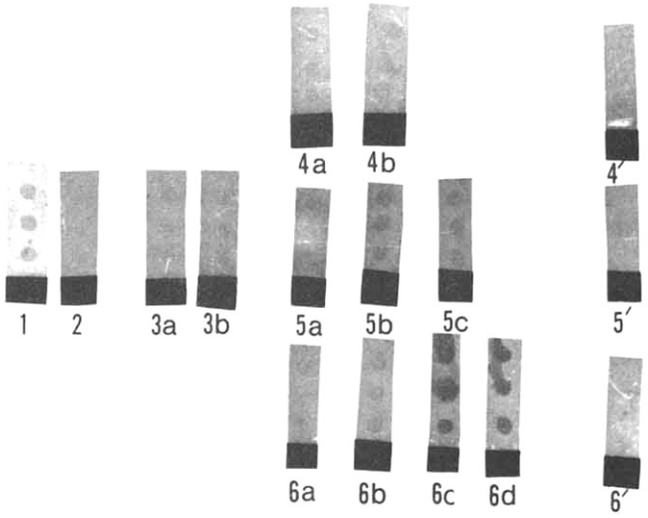

Figure 1. Determination of hCG by indium slide immunoassay. Indium slide 1 was spotted as described in Materials and Methods with purified monoclonal anti-hCG antibody (P_3W_{80}), rinsed, and dried, revealing the adsorbed protein as darkened spots. Slide 2 represents the masking of the monoclonal antibody (P_3W_{80}) with 2% normal goat serum. After masking, slide 3a was incubated with 1 μg of hCG/ml, and 3b with 5 μg of hCG/ml showing the reappearance of spots without any amplification. Slides in rows 4, 5, and 6 represent different amplification procedures employed after incubation with varying amounts of hCG; amplification with monoclonal anti-α-hCG antibody ($P_2 2_3$), slide 4a (500 ng of hCG/ml), 4b (1 μg of hCG/ml); polyclonal anti-hCG antibody, 5a (100 ng of hCG/ml), 5b (500 ng of hCG/ml), 5c (1 μg of hCG/ml); monoclonal anti-α-hCG antibody-HRP conjugate, 6a (1 ng of hCG/ml), 6b (10 ng of hCG/ml), 6c (50 ng of hCG/ml), and 6d (100 ng of hCG/ml), respectively. Slides 4′, 5′, and 6′ represent respective amplified negative controls.

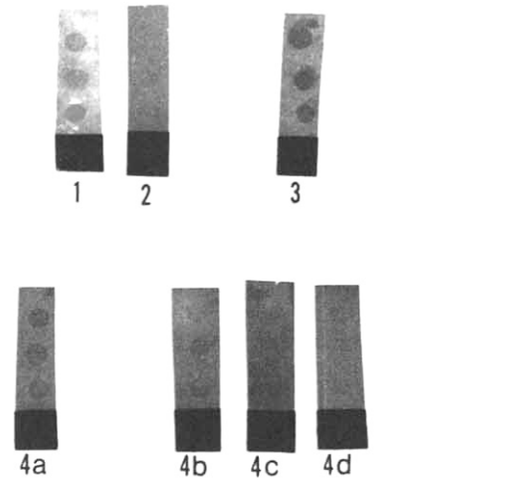

Figure 2. Determination of anti-hCG antibody against specific determinant on hCG involved in bioactivity of the hormone in sera of women immunized with hCG-based contraceptive vaccine. Indium slides 1 and 2 correspond to the slides 1 and 2 as described in Figure 1. After masking, slides were co-incubated with 50 ng of hCG and 1/20 dilution of women's sera followed by reaction with monoclonal anti-α-hCG antibody-HRP conjugate. Slide 4a represents the slide incubated with preimmune sera, 4b with sera having low (~30 ng of hCG binding capacity/ml), 4c having moderate (~100 ng of hCG binding capacity/ml), and 4d having high (~300 ng hCG of binding capacity/ml) anti-hCG antibody titers, respectively. Slide 3 represents positive control, i.e., without women's serum.

noninterference of the serum constituents in the assay (Fig. 2). Gradual decrease in the spot intensity was observed when the indium slides were incubated with pooled immune sera having low (~30 ng of hCG binding capacity/ml), moderate (~100 ng of hCG binding capacity/ml), or high (~300 ng of hCG binding capacity/ml) anti-hCG antibody titer, respectively, as shown in Figure 2.

Discussion

The feasibility of using the indium slide immunoassay for the detection of hCG has been investigated. The whole assay can be completed in less than 3 hr and does not require any sophisticated equipment. Without the use of any amplification step, the sensitivity of the assay was low, i.e., 1 μg of hCG/ml. The end point of the assay is difficult to assess and is subjective, since at the lowest limit of the assay the positive signal is weak (Fig. 1, slide 3a). Antibody is truly present because we could show amplification of the test strips, whereas the negative strips (Fig. 1, slides 4′, 5, and 6′) did not show any further darkening. Amplification with monoclonal anti-hCG antibody could extend the sensitivity by 2-fold. The polyclonal anti-hCG antibody, as compared with monoclonal antibody, was more efficient in increasing the sensitivity (10-fold versus 2-fold), with a lower limit of 100 ng/ml of hCG (Fig. 1, slide 5a). The sensitivity of the indium slide immunoassay employing polyclonal anti-hCG antibody for hCG detection (100 ng of hCG/ml, i.e., 1 IU/ml) was comparable to the currently used agglutination assays. Sensitivity of the indium slide immunoassay could be further extended to 1 ng of hCG/ml (Fig. 1, slide 6a), when enzyme-conjugated monoclonal antibody followed by DAB reaction was used for amplification. The sensitivity of 1 ng of hCG/ml achieved in the indium slide immunoassay compares favorably with the monoclonal antibody-based sandwich enzyme immunoassay employing microtitration plate (1 ng of hCG/ml) (4) or polystyrene ball (7.8 ng of hCG/ml) (16). The indium slide immunoassay, however, has advantages over using microtitration plate in that (i) it requires less time to coat the indium slide than a microtitration plate and (ii) the end result can be read visually. We report three different antiserum reagents to amplify the initial reaction. The polyclonal anti-hCG antibody is more practical than the monoclonal anti-hCG and is simpler and faster than using the enzyme-conjugated reagent. The sensitivity and "readability" are the greatest with the enzyme-amplified assay. The indium slide assay is an adaptable test depending on the purpose of the assay (i.e., simplicity, speed, and sensitivity required). At the moment, a laboratory setting is still required for the performance of the assay.

As a special application, the indium slide immunoassay can also be used to monitor particular anti-

hCG-neutralizing antibodies in women immunized with contraceptive vaccine. Certain of these antibodies recognize a specific determinant on hCG defined by monoclonal antibody, P_3W_{80}, known to neutralize hCG bioactivity. The preliminary results with P_3W_{80} as a model antibody indicate that the indium slide immunoassay can be used for this purpose. We realize that this assay would not detect other antibodies that neutralize hCG via other sites on the molecule, but it is more informative than measuring total antibody to hCG. In the present assay, preimmune and immune sera from women were co-incubated with 50 ng of hCG (based on calculation of the upper limit of hCG levels during early pregnancy). This, in our experience, is the amount of hCG the immune sera should neutralize for a vaccine to be successful in preventing pregnancy. The assay would, therefore, detect the unbound bioactive component of hCG. If a strong spot is seen, it means that the serum has a poor anticontraceptive effect. If no spot is seen, the neutralizing ability of the test serum is strong. However, the assay may have an inherent drawback inasmuch as the polyclonal antibody present in immunized women sera could nonspecifically hinder the binding sites on hCG for the second monoclonal anti-hCG antibody (P_22_3)-HRP conjugate used to show the inhibition in the binding of hCG to indium slide. Pooled sera were used for these preliminary data, therefore additional work is required to apply the method to routine use. Practical utility of such an assay must wait for the Phase II clinical trial studies of the hCG immunocontraceptive vaccine, in which the results in the indium slide immunoassay can be compared with the duration of infertility.

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