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An avidin–biotin micro-enzymeimmunoassay for monkey chorionic gonadotrophin*

R. Chakrabarti and A. Jagannadha Rao

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Summary. The avidin–biotin micro-enzymeimmunoassay used antiserum to the β-subunit of sheep LH, biotinylated hCG as tracer and pooled serum from pregnant monkeys as a reference preparation of monkey CG. The assay was validated for specificity, by checking serum samples known to contain high quantities of LH, and false positive results were eliminated to a great extent by prior heating of serum samples at 60°C for 30 min. In 45 bonnet monkeys the test gave 10 true positives, 34 true negatives and 1 false positive, i.e. an accuracy of 97%. The serum profiles of CG, measured by the EIA, in bonnet, cynomolgus and rhesus monkeys during early pregnancy were very similar to those reported for bonnet and rhesus monkeys when a liquid-phase radioimmunoassay was used.

Introduction

The presence of chorionic gonadotrophin (CG) in serum or urine has been traditionally used as an index of pregnancy in primates. Although its presence in urine or serum of several non-human primates has been reported (Hodgen, 1980), only human CG has been well characterized. However, it has not been possible to use even the most sensitive hCG immunoassays for diagnosis of pregnancy in macaques, due to an apparent lack of cross-reactivity of antisera to hCG or its beta subunit with monkey CG (Hodgen, 1980). At present the available haemagglutination inhibition test (Hodgen & Ross, 1974) and radioimmunoassay (Hodgen *et al.*, 1974; Jagannadha Rao *et al*, 1984) for monkey CG are based on the partial cross-reactivity of certain batches of rabbit antisera to the beta subunit of sheep luteinizing hormone (LH) with monkey CG. Although the liquid-phase radioimmunoassay reported for rhesus monkey CG (Hodgen *et al.*, 1974) is quite sensitive, the liquid-phase (Jagannadha Rao *et al.*, 1984) and solid-phase (Chakrabarti *et al.*, 1985) radioimmunoassays used for bonnet monkey CG were not sensitive enough to diagnose pregnancy during very early stages of gestation. The avidin–biotin system has been used to amplify the sensitivity of enzyme immunoassays (Guesdon *et al.*, 1979, Rappuoli *et al.*, 1981). In the present communication we report the development and application of a micro-enzymeimmunoassay based on the avidin–biotin system for CG of the bonnet monkey.

Materials and Methods

Hormones and chemicals. Highly purified β-subunit of sheep LH was a gift from Dr C. H. Li (Molecular Endocrinology Laboratory, University of California, San Francisco, U.S.A.) and Dr M. R. Sairam (Clinical Research Institute of Montreal, Montreal, Canada). Highly purified hCG, batch No. CR 123, (12 000 i.u./mg) was generously provided by NIAMDD, Bethesda, MD, U.S.A. Antiserum to sheep LH β-subunit was raised and characterized as described by Jagannadha Rao *et al.* (1984). Bovine serum albumin (BSA), RIA grade; bovine gamma globulin, Cohn fraction II; protein A; biotin-*N*-hydroxy succinimide ester; horseraddish peroxidase–avidin conjugate; Tween-20; and sodium thiocyanate were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Sephadex G-25 and CNBr-Sepharose 4B

*Reprint requests to Dr A. Jagannadha Rao.

were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydrogen peroxide (30% v/v) was from Glaxo Laboratories, Bombay; and O-phenylene diamine was from Ferak, West Berlin, FRG. Polyvinylchloride (PVC) micro-titre plates (96 wells, U-shaped) were from Dynatech Company, Alexandria, VA, U.S.A.

Animals and blood sampling. Adult cyclic female bonnet monkeys (*Macaca radiata*) weighing 5–6 kg were used in the study and husbandry was as previously described (Prahalada *et al.*, 1975). The day on which vaginal bleeding was first noticed was considered as Day 1 of the menstrual cycle. Female bonnet monkeys were housed in the cages of selected proven fertile males from Days 9 to 14 of the cycle. Female monkeys in this colony exhibit peak values of serum oestradiol-17β on Day 9 or 10, the ovulatory surge of LH occurring within 24 h of this surge (Ramasharma *et al.*, 1978). Because the length of follicular phase can be variable, the exact day on which fertilization occurs may be on any one of Days 9–14 of the cycle. For convenience in this study, therefore, Day 12 of the fertile cycle has been considered as Day 1 of pregnancy. Blood samples were collected, between 09:00 and 10:00 h, from unanaesthetized animals using vacutainer tubes. Serum was separated within 12 h and stored at −20°C until further processing. Serum samples from pregnant cynomolgus and rhesus monkeys were generously provided by Dr A. G. Hendrickx, California Primate Research Center, Davis, CA, U.S.A., and Dr D. L. Hess, Oregon Regional Primate Research Center, Beaverton, OR, U.S.A., respectively.

Isolation of immunoglobulin G (IgG). IgG from the antiserum to sheep LH β-subunit was isolated by affinity chromatography using a CNBr-Sepharose 4B-protein A column. The affinity column was prepared according to the supplier's instructions. In brief, 1 ml antiserum was dialysed against phosphate-buffered saline (PBS, 0·01 M, pH 7·4: 0·89 g $Na_2HPO_4.2H_2O$, 0·144 g $KH_2PO_4.2H_2O$ and 8·7 g NaCl/l double-distilled water overnight at 4°C and centrifuged at 10 000 *g* for 20 min at 4°C. The clear supernatant was passed through the affinity column equilibrated with PBS, pH 7·4. The process was repeated 6 times and IgG was eluted from the column using 3 M-sodium thiocyanate in PBS, and 1-ml fractions were collected. Appropriate fractions were pooled and dialysed against PBS overnight at 4°C and protein in the dialysed solution was estimated by the method of Lowry *et al.* (1951) using bovine gamma globulin as a standard. Immunoactivity of the isolated IgG was checked by testing its ability to bind ^{125}I-labelled hCG. Highly purified hCG was iodinated according to the procedure of Greenwood *et al.* (1963) and specific activity of the iodinated hCG ranged from 40 to 50 μCi per μg.

Biotinylation of hCG. Biotinylation of hCG was done according to the method of Rappuoli *et al.* (1981). Biotin *N*-hydroxy succinimide ester (10 μl) dissolved in distilled dimethyl formamide (5 mg/ml) was added to 0·1 ml carbonate buffer (0·1 M, pH 8·5) containing 500 μg hCG. After incubation for 18 h at room temperature, the mixture was fractionated on a Sephadex G-25 column equilibrated with 20 mM-ammonium bicarbonate. The void volume peak was lyophilized and residual amino groups were estimated by the 2,4,6-trinitrobenzene sulphonic acid method of Snyder & Sobocinski (1975). Immunoreactivity of biotinylated hCG was estimated by a double-antibody radioimmunoassay using antiserum to sheep LH β-subunit and ^{125}I-labelled hCG.

Reference preparation of monkey CG. As no reference preparation for monkey CG was available for dose interpolation in the immunoassay, an ad-hoc reference preparation of bonnet monkey CG was prepared from a pool of serum from pregnant bonnet monkeys collected from Day 28 (Day 17 of gestation) to Day 40 (Day 29 of gestation) of a fertile cycle. The bioactivity of CG in the pool of pregnancy serum was estimated by the mouse uterine weight bioassay method (Tullner & Hertz, 1966), using hCG as the standard. The dose–response curves for hCG (3 doses) and pooled pregnancy serum (3 doses) did not significantly differ from each other (*t* test). The serum pool had a biological activity of 1·8 i.u. hCG (CR 123) equivalents per ml and was used as the laboratory standard preparation of monkey CG.

Buffers for the EIA. All the buffers used in the EIA were prepared using double-distilled water. The coating buffer was 0·1 M-sodium carbonate–bicarbonate buffer, pH 9·6. The rinse buffer was 0·01 M-PBS, pH 7·4, containing 0·05% Tween-20. The buffer for diluting horseraddish peroxidase–avidin and biotinylated hCG was PBS, pH 7·4, containing 0·5% BSA. The buffer for enzyme substrate reactions was 0·2 M-potassium phosphate buffer, pH 7·0.

Avidin–biotin micro-enzymeimmunoassay procedure. Antiserum samples (0·1 ml) of the IgG β-subunit of sheep LH (2·5 μg/ml coating buffer) were added to PVC micro-titre wells and left for 90 min at 37°C in an humidified incubator. The wells were then washed 3 times with rinse buffer. Serum samples, hCG or the laboratory standard preparation of monkey CG (100 μl) appropriately diluted with heat-treated (at 60°C for 30 min) normal monkey serum were added to each well. The plates were incubated at 37°C for 2·5 h in an humidified incubator, and then washed 3 times with rinse buffer and 100 μl biotinylated hCG (20 ng/ml diluent buffer) were added to each well and incubation continued for 30 min at 37°C. This was followed by a wash with rinse buffer and incubation with 100 μl of horseraddish peroxidase–avidin conjugate (1 μg/ml diluent buffer) for 10 min at 37°C. After washing the wells, the activity of the immobilized enzyme was monitored by addition of 100 μl of freshly prepared substrate solution (1 mg O-phenylene diamine/ml and 0·006% H_2O_2 in substrate buffer) and incubating for 20 min at room temperature in the dark. The enzyme reaction was terminated by adding 160 μl 2 N-HCl and the absorbance was read at 492 nm in a spectrophotometer. Wells incubated with an excess of hCG (50 ng/well) served as non-specific control and wells incubated only with biotinylated hCG served as the specific binding control (B_0).

Optimization of assay conditions. The optimum conditions (individual reagent concentration, volume, temperature and duration of incubation) for the assay procedure were determined by varying each condition at a time, keeping the others constant.

Test for specificity. The relative specificity of the assay was established by assaying serum samples from cyclic (midcycle, Day 10 to Day 16), ovariectomized, lactating and pregnant monkeys (bonnet, rhesus and cynomolgus). In addition, pituitary extracts (one pituitary/ml PBS) and placental extracts from bonnet monkeys were also tested in the assay.

Statistical analysis. The dose–response data for the laboratory standard preparation of monkey CG and hCG were plotted on a logit–log scale. The logit (y) was calculated as Logit (y) = $\ln[B/B_0/1-(B/B_0)]$ where B_0 is value at zero concentration of the CG preparations and B is the value in the presence of the CG preparations. The best-fitted lines were obtained by simple linear regression analysis of the data and the regression coefficients and correlation coefficients obtained were validated by Student's t test (Zar, 1974). The significance of difference in readings between zero concentration and the lowest concentration of the laboratory standard preparation of monkey CG was checked by Student's t test (Zar, 1974).

Results

Isolation of IgG

From 1 ml antiserum to the β-subunit of sheep LH, 4·5 mg IgG was obtained and it retained its full binding activity for ^{125}I-labelled hCG as determined by double-antibody radioimmunoassay.

Biotinylation of hCG

Analysis for the residual amino groups after biotinylation revealed that 64% of the total exposed amino groups were biotinylated. The biotinylated hCG retained its full immunoreactivity as determined by radioimmunoassay and no loss of activity was noticed even after 18 months of storage at −20°C.

Dose–response curves for hCG and the laboratory standard preparation of monkey CG

As can be seen from Fig. 1, the dose–response curves for these substances were not parallel and the difference between the slopes was statistically significant ($P < 0{\cdot}05$). The sensitivity of the assay for monkey CG, i.e. the lowest concentration of monkey CG which could be distinguished from zero dose at 95% confidence level, was 40 munits/ml serum. This was equivalent to 3·3 ng hCG (CR 123) with a biological activity of 12 000 i.u./mg.

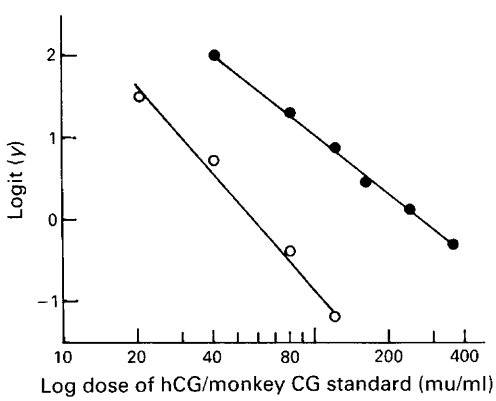

Fig. 1. Standard inhibition plot for the laboratory standard preparation of monkey CG (●) [n = 10] and hCG (○) [n = 5]. For the standard, logit (y) = 3·45 −2·45 (log x); slope = −2·45 ($P < 0{\cdot}001$), r = −0·998 ($P < 0{\cdot}001$). For the hCG, logit (y) = 2·63 −3·45 (log x); slope = −3·45 ($P < 0{\cdot}01$), r = −0·994 ($P < 0{\cdot}01$)

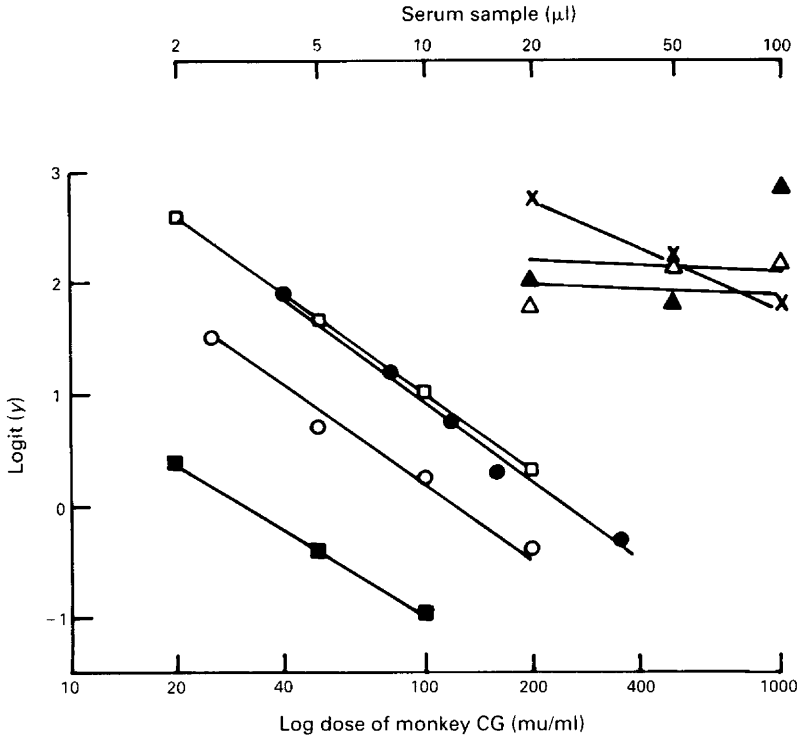

Fig. 2. Inhibition lines for the laboratory standard preparation of monkey CG (●) and various quantities of pooled serum, ranging from 2 to 100 µl, of midcyclic (△) ovariectomized (×), lactating (▲) and early pregnant (○) bonnet monkeys and, early pregnant cynomolgus (■) and rhesus (□) monkeys.

Suitability of the reference preparation and relative specificity of the asssay

It can be seen from the data presented in Fig. 2. that increasing volumes of pregnant monkey serum (bonnet, rhesus and cynomolgus) produced dose–response curves parallel to that for the laboratory standard preparation of monkey CG, indicating that this standard is a suitable reference preparation for assay of monkey CG in serum. Since LH and hCG share considerable structural homology, and the antisera to each cross-react with the other, it is possible that pituitary LH in the serum may interfere in the monkey CG assay. The data in Fig. 2 indicate that inclusion of up to 100 µl serum from ovariectomized or midcycle monkeys (known to have high serum concentrations of LH) or lactating monkeys (known to have high concentrations of prolactin) did not result in any significant inhibition in the assay. Also, an aqueous extract of bonnet monkey pituitaries produced very negligible inhibition. In contrast, monkey placental extract (Day 55 of gestation) produced a dose-dependent inhibition which was parallel to that produced by the laboratory standard preparation (Fig. 3). These results therefore establish that the assay is relatively specific for monkey CG in that the amounts of LH-like molecules at 15–19 days after fertilization are well above the levels of potentially cross-reacting substances.

Within- and between-assay variation

Within-assay coefficient of variation for monkey CG values in serum pools containing 158 ± 14 and 270 ± 43 munits/ml were 8% and 16%, respectively. The between-assay coefficient of variation for 6 different concentrations of the standard preparation (40–360 munits/ml) ranged from 7 to 16%.

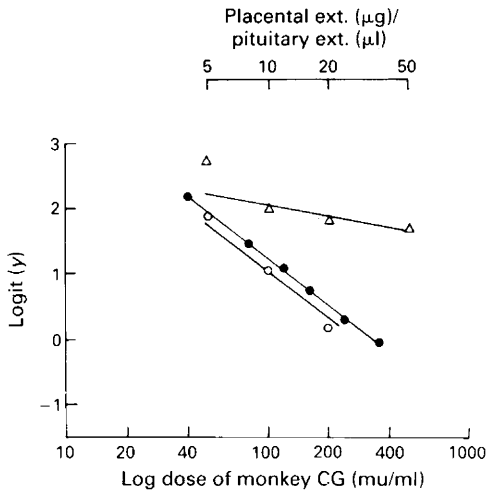

Fig. 3. Inhibition lines for the laboratory standard preparation of monkey CG (●) bonnet monkey pituitary extract (△, 5–50 μl of extract of one pituitary per ml PBS) and mid-gestation bonnet monkey placental extract (○, 5–20 μg lyophilized powder).

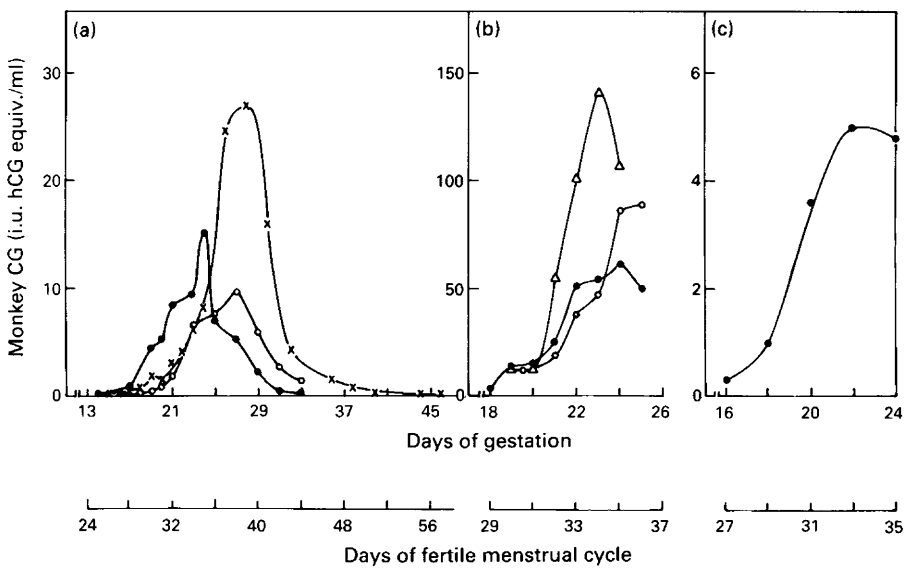

Fig. 4. Serum immunoreactive monkey CG during a fertile menstrual cycle in (a) 3 bonnet monkeys (*M. radiata*) (b) 3 cynomolgus monkeys (*M. fascicularis*), and (c) rhesus monkeys (*M. mulatta*: serum samples were pooled from 3–4 animals on individual days of gestation).

Application of the assay for diagnosis of pregnancy in the bonnet monkey

Serum samples (100 μl) collected on alternate days from Day 24 (Day 13 of gestation) to Day 30 (Day 19 of gestation) of a fertile cycle were tested. Before use, all the serum samples were heated at 60°C for 30 min to eliminate the serum interference in the assay. Out of a total 45 animals tested for the presence of CG in the serum, 10 were truly positive, 34 were truly negative and one gave a false positive result. The test results were confirmed by rectal palpation on Day 35 of gestation and also

by delivery of live young. The percentage accuracy was therefore 97–100%. The earliest day on which pregnancy could be diagnosed with certainty was the 26th day of a fertile cycle which corresponded to Day 15 of gestation, which is just 5–6 days after implantation in the monkey (Hendrickx & Enders, 1980).

Serum concentrations of CG in bonnet, cynomolgus and rhesus monkeys

The test was used to measure serum concentrations of CG in 3 bonnet monkeys, 3 cynomolgus monkeys and in pooled serum of rhesus monkeys (Fig. 4). In all the bonnet monkeys the earliest day on which CG could be detected was once again Day 26 of the fertile cycle (Day 15 of gestation), the concentration ranging from 0·05 to 0·09 i.u./ml during this period. Peak values were observed between Days 35 and 39 of the fertile cycle (Days 24–28 of gestation), the values ranging from 9·6 to 29·9 i.u./ml. Values then declined rapidly and reached low values by Days 50–55 of the fertile cycle (Days 39–44 of gestation). The serum CG profiles during early pregnancy in the 3 cynomolgus monkeys were very similar to those of the bonnet monkeys, although the serum values were much higher. Although samples from all days during early pregnancy have not been analysed, the available results indicate that the profile of CG in rhesus monkeys is similar to that in bonnet and cynomolgus monkeys.

Discussion

The avidin–biotin micro-EIA for monkey CG reported here has certain advantages over the liquid- and solid-phase radioimmunoassays reported earlier by us. These include improved sensitivity (the minimum detectable quantity of monkey CG in the micro-EIA is 40 munits/ml as compared to 400 munits/ml in the liquid-phase radioimmunoassay), dispensation of the use of radiolabelled ligand, stability and uniformity of the enzyme immunoassay reagents (the specific activity of ^{125}I-labelled hCG can vary depending upon the batch of iodine and iodination conditions) and simplicity in operation. The improved sensitivity of an enzyme immunoassay using the avidin–biotin system is mainly due to three factors. Firstly, the problem of steric hindrance in antigen–antibody interaction is minimized by the use of biotinylated antigen instead of antigen–enzyme conjugate. Secondly, more biotin molecules than enzyme molecules can be linked to a single antigen molecule. Thirdly, since the horseraddish peroxidase–avidin ratio in a commercially available conjugate is 2:1, the number of enzyme molecules bound to immobilized antibody–biotinylated hCG complex is doubled. The sensitivity of an immunoassay depends mainly on the affinity of the antibody for the antigen under consideration. The liquid-phase radioimmunoassay for rhesus CG described by Hodgen *et al.* (1974) is much more sensitive than the one described by us (Jagannadha Rao *et al.*, 1984) for bonnet monkey CG, indicating that the LH β-subunit antibody used by us has relatively lower affinity for monkey CG. However, in the present study, even with a relatively low affinity antibody, we have been able to improve the sensitivity considerably by coupling it with the biotin–avidin system, and the sensitivity could be further improved with the antibody used by Hodgen *et al.* (1974).

With the EIA described in this study, pregnancy in the bonnet monkey can be diagnosed with a high degree of accuracy on 26th day of a fertile cycle (15th day of gestation) which is 4 days earlier than by the liquid-phase radioimmunoassay. However, it was not possible to demonstrate the limit of detectability (earliest day on which pregnancy could be diagnosed) in rhesus and cynomolgus monkeys due to lack of serum samples on all days, particularly during the early period. The limit of detectability is variable amongst non-human primate species, for example, the period over which CG could be detected by the haemagglutination inhibition test is different between cynomolgus and

rhesus monkeys (Hodgen *et al.*, 1977). Peak values of CG are about 5 i.u./ml in rhesus monkeys and 150 i.u./ml serum in cynomolgus monkeys. In contrast to the high levels and prolonged duration of presence of CG in humans, CG concentrations are extremely low and are detectable over a very short duration in the monkeys. Considering this fact, the present improvement in sensitivity which permits diagnosis of pregnancy 4 days earlier is of considerable importance. The development of a reliable, quick and sensitive assay to diagnose early pregnancy in non-human primates can be of immense help in economic use of monkeys and in designing experiments. The assay reported here has been validated for specificity by assaying serum samples containing high LH as well as pituitary extract. The maximum volume of serum samples used in the assay is 100 µl and this amount of serum containing a high quantity of LH (2 µg per ml) did not show any inhibition in the assay. These results suggest that this assay is relatively specific for monkey CG without any interference from serum gonadotrophins of pituitary origin.

One of the main problems generally encountered in the application of a monkey CG immunoassay (haemagglutination inhibition test or radioimmunoassay) has been the high incidence of false positive results. Initially, we encountered a very high percentage of false positive results for pregnancy diagnosis in bonnet monkeys, and the interference was considered to be due to proteases present in the serum. However, a detailed study, wherein a number of protease inhibitors were included in the assay, ruled out this possibility. The false positive results were obtained only with fresh serum samples or serum samples stored at −70°C. Samples stored at room temperature or at 4°C for a period of 7 days or more did not show any false positive reaction. This suggested that the false positive results could be due to a heat-labile material, and in the present EIA, we were able to eliminate the false positive reactions to a considerable extent by heating serum samples at 60°C for 30 min. While inclusion of fresh monkey serum resulted in 74% inhibition, the same serum after heat treatment caused no inhibition. In contrast, similar heat treatment had no effect on the immunoreactivity of the laboratory standard preparation of monkey CG. The efficacy of this treatment is evident from the fact that only one false positive result (2·9%) was encountered and even this could have been due to very early termination of pregnancy or the presence of cross-reacting material in the serum. The overall accuracy with which pregnancy could be detected was over 97% and this is comparable to the accuracy reported using radioreceptor assay for CG in rhesus and cynomolgus monkeys (Booher *et al.*, 1983). Considering that this is the first report of a heterologous enzymeimmunoassay for monkey CG, which is still not available in a purified state, this is a significant improvement over the existing assays.

The results presented in this study also indicate that the serum CG profiles determined by EIA for bonnet monkeys are comparable to those reported earlier using liquid-phase radioimmunoassay. However, it is not possible to make direct comparison between the absolute values of the serum CG determined by EIA and RIA, as the reference preparations used in the two assays are quite different. The dose–response curves of hCG and monkey CG are not parallel and the use of a standard preparation for monkey CG as a reference preparation for quantitation of CG is more suitable for accurate estimation of monkey CG values in the serum. The fact that we have been able to use the bonnet monkey CG EIA for determination of CG levels in cynomolgus and rhesus monkeys, and the report that antiserum to the β-subunit of sheep LH cross-reacts with CG from several non-human primates (Hodgen, 1980), suggests that it should be possible to use this avidin–biotin micro-EIA method for diagnosis of pregnancy in other non-human primates.

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