THE EXCRETION OF HUMAN CHORIONIC GONADOTROPHIN IN NORMAL PREGNANCY.
AN IMMUNOLOGICAL INVESTIGATION

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SUMMARY

The haemagglutination-inhibition test was investigated as an immunological method for the assay of human chorionic gonadotrophin (HCG). The preparation of a potent antiserum to HCG is described. The results of the assay of HCG during normal pregnancy in ten patients are compared with the results of immunological assays from two Swedish laboratories. The mean values and the 95% confidence limits for the excretion of HCG from the 10th week of normal pregnancy are calculated.

INTRODUCTION

The use of immunological methods for estimating the levels of hormones in biological fluids was first described by Arquilla & Stavitsky (1956) for insulin; in 1958 Read & Stone applied the method to growth hormone. Three different techniques for the assay of human chorionic gonadotrophin (HCG) were reported in 1960: the complement-fixation test was developed by Brody & Carlström, the precipitin test by McKean, and the haemagglutination-inhibition test by Swierczynska & Samochowiec and by Wide & Gemzell. Later, Wide (1962) gave more details of the last method.

So far only two groups of workers have reported on the use of immunological techniques to determine the pattern of HCG production throughout normal pregnancy. Brody & Carlström (1961, 1962) measured HCG in serum samples from pregnant women and found that the mean values followed a biphasic curve with two peaks, the higher one at 10–12 weeks and the smaller one at 34 weeks. Wide (1962) and Wide & Gemzell (1962) measured the HCG content of early morning specimens of urine throughout pregnancy by the haemagglutination-inhibition method. They obtained a monophasic curve with the peak at 10 weeks. The levels of HCG suggested by both methods tend, throughout pregnancy, to be higher than those indicated by biological assays. These discrepancies justify a further investigation of the haemagglutination-inhibition test for estimating the amount of HCG in the urine during pregnancy.

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mATERIALS AND METHODS

Collection of samples

Early morning specimens of urine were collected daily from patients with normal pregnancies. Wilson, Albert & Randall (1949) showed that the fluctuations in the amount of HCG excreted during 24 hr. are not due to variable renal clearance but to alterations in the rate of production of the hormone. Using the haemagglutination-inhibition technique to assay HCG in urine, Wide (1962) showed conclusively that there is no significant difference between the results of the assays performed on early morning samples and assays carried out on 24 hr. collections of urine. In view of this conclusion and because of the practical difficulties of obtaining complete 24 hr. collections, first morning specimens of urine were used throughout this investigation. The samples were stored at 0° until tested. Immediately before testing they were passed through Whatman No. 1 filter paper.

Preparation of antiserum

Adult rabbits were immunized with HCG (Pregnyl, Organon) in an adjuvant composed of aluminium phosphate gel in mineral oil. The material for injection was prepared under strict aseptic conditions, by dissolving 12000 i.u. HCG in 1 ml. of saline and mixing it with 4 ml. of 3-4% aluminium phosphate suspension; 4 ml. of Bayol 55, a light paraffin oil, and 1 ml. of Arlacel A were then added to make an emulsion. Aluminium phosphate gel was used rather than Freund adjuvants because Hayward & Augustin (1957) showed that it results in a high titre of antibody. This expectation proved correct and antiserum titres of up to 1/65000 were obtained.

Commercial preparations of HCG, extracted from pooled pregnancy urine, are not highly purified and contain several antigens. According to Kabat (1958), aluminium phosphate has the added advantage that it is less likely to produce a strong antibody response to minor components of an antigenic complex than the Freund adjuvants. That the technique used in this study does stimulate the production of antibodies to HCG was confirmed by gel diffusion and immunoelectrophoretic experiments.

Immunization technique

The immunization technique was based on the method described by Hayward & Augustin (1957). Each rabbit received 10 ml. of the HCG-adjuvant mixture, 5 ml. i.p. and the remainder s.c. and i.m. One treatment with 10 ml. every 3 months proved sufficient to maintain a high titre of antibody in the serum.

The rabbits were bled at intervals of 2-3 weeks; the serum was stored at -10°. All sera used, from untreated rabbits as well as those containing antibodies to HCG, were absorbed with washed sheep red cells to remove any naturally occurring anti-sheep red cell agglutinins.

Preparation of HCG-sensitized cells

The sheep red cells used for this part of the test must be formalinized. The method of sensitizing the cells with HCG was based on the method of Wide & Gemzell (1960). The only modifications introduced were that 1/20000 tannic acid was used for tanning the cells, and 100 i.u. HCG/ml. for sensitizing them. The composition of the tannic acid and buffer solutions is critical; they must be freshly prepared.
Absorption of antiserum with urinary proteins

Antiserum was absorbed twice with 10 mg./ml. of dry extract of male urinary protein which was prepared by the kaolin/acetone method of extraction. The antiserum was stored at 0° for 72 hr. after each absorption, before the precipitate was removed. That the antiserum was adequately absorbed was demonstrated by the complete removal of the urinary protein precipitin lines as distinct from the specific HCG precipitin lines when the antiserum was allowed to react with HCG and urinary protein in gel diffusion and immunoelectrophoresis experiments. This aspect of the investigation has been described in detail elsewhere (Connon, 1963), but here it can be stated that the absorption process did not reduce the titres of the antiserum.

Haemagglutination patterns

Perspex agglutination trays (M.R.C. pattern) were used for the assays. The degrees of agglutination which occurred were classified into five groups according to the following criteria: (0), no agglutination ('button', small without a centre); (1), no agglutination ('button' with small centre); (2), partial agglutination (definite rim of cells with widish centre to button); (3), almost complete agglutination (some agglutination with a narrow rim of cells around the mat or shield); (4), complete agglutination (cells form a smooth mat or shield which fills the bottom of the 'tube'). In interpreting the results the critical point taken was between group 2 and 3. Accordingly, patterns 3 and 4 were accepted as positive haemagglutination but all others were classified as negative haemagglutination.

Assay of HCG

The haemagglutination-inhibition test employed was that described by Wide & Gemzell (1960) with slight modification. Wide, Roos & Gemzell (1961) stated that the haemagglutination-inhibition test for HCG could also be used to measure luteinizing hormone. In the preliminary stages of this investigation it was found that when an extract of postmenopausal urinary gonadotrophin (HMG) was substituted for HCG, concentrations as high as 8 mg. HMG/ml. failed to inhibit haemagglutination when a 1/200 dilution of antiserum was used. Indeed, in order to titrate HMG it was necessary to increase the dilution of antiserum to 1/800. Since the experiments were designed to assay HCG and not HMG, it was decided to use antiserum diluted to 1/200 routinely thus avoiding any interference from urinary gonadotrophins of pituitary origin. This adherence to a constant dilution proved satisfactory and the particular dilution was sufficiently sensitive to detect amounts of HCG ranging from 0·48 to 1·9 i.u./ml. of urine. Absorption of antiserum with urinary protein did not alter the values obtained for the amount of HCG in urine; this indicates that the antibodies to inert urinary proteins in the antiserum do not affect the assay of HCG.

Five control tests were carried out as part of each experiment, as follows:

1. A haemagglutination-inhibition test, using the last three washings of the HCG-sensitized cells as the antigen, to make sure that the HCG was firmly adherent to the cells and that the cell suspension did not contain free HCG.
2. A haemagglutination-inhibition test with normal rabbit serum instead of antiserum to HCG to exclude false agglutination of the sensitized cells.

3. A haemagglutination-inhibition test with urine from a pregnant woman as the antigen and normal rabbit serum instead of antiserum to HCG. This was done to eliminate the possibility of false agglutination of the cells in the presence of non-specific constituents of the urine.

4. A haemagglutination-inhibition test using urine from a pregnant woman as the antigen and adding formalinized tannic acid-treated erythrocytes instead of HCG-sensitized cells. This again was to exclude false agglutination of the cells as a result of non-specific factors in the urine.

5. A haemagglutination test with antiserum to HCG and HCG-sensitized cells to establish that the antiserum had the capacity to agglutinate the sensitized cells.

Fig. 1. Urinary HCG concentration during ten normal pregnancies. Each line represents one woman and each dot the average for a week (log scale).

RESULTS

Early morning specimens of urine were obtained from ten women in an early stage of pregnancy. In the majority of cases the urine samples were obtained daily; the total number tested was 1253. All the pregnancies proceeded normally without toxaemia or other diseases. The babies were born alive and were of average weight and the placentas were macroscopically normal. There was a wide fluctuation in the range of HCG excretion, even in individual patients. Weekly averages of the results are shown in Fig. 1. The HCG levels for the first few weeks of pregnancy covered a wide range. The range of values of HCG up to the 9th week of gestation was very great and therefore only assays performed after the 9th week were subjected to detailed analysis. (For statistical methods used see appendix.)

Fig. 2 shows the weekly mean values for urinary HCG excretions and the 95% confidence limits.

The mean values for HCG excretion in normal pregnancy ranged from 65 000 i.u./l. urine at 10 weeks, to 40 000 i.u./l. at 20 weeks, 25 000 i.u./l. at 30 weeks, and 15 000 i.u./l. at 40 weeks. There was a rise in the level of HCG excreted between the 32nd and 36th weeks of gestation.
Chorionic gonadotrophin in pregnancy

Of the 1190 assays analysed statistically, only 49 fell outside the 95% confidence limits shown in Fig. 2; twenty-five fell above and twenty-four below these limits, corresponding to 4.1% of the total number of observations.

Fig. 2. The weekly mean values and 95% confidence limits (dots) for urinary HCG concentrations during ten normal pregnancies (log scale).

**DISCUSSION**

The main feature of the results shown in Fig. 2 is that the mean values of HCG show a gradual fall from the 10th week of pregnancy and that the level never falls below 15000 i.u. HCG/l. urine. This is a higher level than that generally indicated by biological assays (Jones, Delfs & Stran, 1944; Loraine, 1949; Wilson et al. 1949). The biological assays were performed on 24-hr. samples of urine rather than on morning specimens, but this is unlikely to account for the difference.

There was a definite rise in the excretion of HCG at the 32nd week of pregnancy (Fig. 2) which gives a biphasic appearance to the curve, allowing for the fact that the first portion of the curve, up to the 10th week of gestation, is not included in this figure. These results are similar to the biphasic curve obtained by Jones et al. (1944).

There is as yet no proof that the immunological techniques measure exactly, or are likely to measure the same substance, as do biological methods. The latter, for example, do not distinguish between chorionic gonadotrophin and pituitary luteinizing hormone.

There is no doubt that there is a difference between the biological and the immunological activities of HCG. This was demonstrated in this laboratory by heating a known sample of HCG at 80° for 1 hr. and then testing it for its relative activities. Most of its biological activity was destroyed but its immunological activity, as determined by the haemagglutination-inhibition test, was unaltered. This may be due to denaturation of the determinant group on the HCG molecule which is responsible for its biological activity but, until more is known about the structure of HCG, this can only be an assumption.

It could be argued that the immunological test measured something other than HCG. To refute this, control experiments with urine from non-pregnant women were done. In no case did such urine inhibit haemagglutination. Moreover, it was possible
to show that the immunological test rapidly becomes negative after parturition. A further group of ten patients provided daily samples of urine from the 38th week of pregnancy until the 10th day of the puerperium. Subjection of these specimens of urine to the haemagglutination-inhibition test clearly showed that the level of HCG fell to zero within 5 ± 3 days after delivery. These results provide proof that the immunological technique measures a substance which is only present during pregnancy and is presumably of placental origin.

Analysis of the results of this investigation produced a curve for the mean HCG excretion (Fig. 2) which is not monophasic like the curve produced by Wide & Gemzell (1962) and Wide (1962), but biphasic like that of Brody & Carlström (1962).

Since this investigation was completed, Mishell, Wide & Gemzell (1963) have published a report comparing the results of the haemagglutination-inhibition test for the assay of HCG in serum with results for HCG in urine. They found it necessary to extract serum with acetone before it could be assayed, but the results were similar to those which they obtained from assays of urine. However, they stressed the point that there is such a wide range in the individual results that a large number of assays must be performed before the level of HCG in serum throughout normal pregnancy can be established.

The results of Mishell et al. (1963) serve to strengthen the need for the study of further cases of normal pregnancy. Even when this has been done, it will be necessary for each laboratory to establish its own range of values for the excretion of HCG throughout normal pregnancy before an immunological assay for HCG can be used as a routine procedure. Only then can departures from the standard findings, which may be associated with pathological conditions of pregnancy, be reliably interpreted.

This investigation was carried out during the tenure of the University of Liverpool Research Fellowship in Obstetrics and Gynaecology from 1961 to 1963.

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APPENDIX

Statistical analysis

Records for an average of seven patients were obtained each week and analyses of variance were made on the variate \( \log_{10} \) HCG concentration for each week. These were single classification analyses of variance with unequal numbers of observations within the subclasses and took the following form:

<table>
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<th>Variation due to</th>
<th>D.F.</th>
<th>Expected value (mean square)</th>
</tr>
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<tr>
<td>Between subjects</td>
<td>( s - 1 )</td>
<td>( \sigma^2 + n^2 \sigma^2 )</td>
</tr>
<tr>
<td>Between days within subjects</td>
<td>( n - s )</td>
<td>( \sigma^2 )</td>
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<tr>
<td>Total</td>
<td>( n - 1 )</td>
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Estimates of the ‘within subject ($\sigma_w^2$)’ and ‘between subjects ($\sigma_s^2$)’ variance components were obtained in the usual way (Williams, 1954). Bartlett’s test of homogeneity was made on the thirty-one estimates of each variance component. This test showed that the estimates of $\sigma_w^2$ were not homogeneous ($\chi^2_{30} = 93.5$) for each of the 31 weeks, but the estimates of $\sigma_s^2$ were homogeneous ($\chi^2_{30} = 38.83$). The estimates of $\sigma_s^2$ were pooled and the mean value was 0.1171. The heterogeneity of the estimates of $\sigma_w^2$ was apparently due to occasional erratic observations, together with a definite reduction in the value of this variance component from week 30 onwards.

Estimation of confidence limits:

Assuming that the assay will usually consist of a single estimate from one subject, the variance of $\log_{10}$ HCG concentration of this estimate is: $\sigma_w^2 + \sigma_s^2$. When more than one determination is made on a patient over a period of a few days, then

$$V(\bar{y}) = \frac{\sigma_w^2}{n} + \sigma_s^2,$$

where $n =$ number of determinations made.

Using the estimated weekly mean concentrations ($\bar{y}_i$), the upper and lower 95 and 99% confidence limits for HCG concentration of a single determination were calculated as

$$\bar{y}_i \pm 2\cdot 0 \sqrt{(\sigma_w^2 + \sigma_s^2)},$$

$$\bar{y}_i \pm 2\cdot 6 \sqrt{(\sigma_w^2 + \sigma_s^2)},$$

where the values of 2.0 and 2.6 correspond to the 5 and 1% values of the statistic $t$. The degrees of freedom appropriate to $t$ cannot be estimated reliably due to non-independence of the estimates of $\sigma_s^2$ but the error involved in taking the values of 2.0 and 2.6 will be almost negligible. The effective degrees of freedom for $t$ are probably greater than 30, since most of the estimates of $\sigma_s^2$ have more than 30 degrees of freedom and the pooled estimates of $\sigma_s^2$ are based on 186 degrees of freedom but these are not independent.

The confidence limits were obtained by graphing the calculated actual limits for each week and drawing a smooth curve through these points. The 95% confidence limits shown in Fig. 2 were obtained from the smooth curve.

REFERENCES


