

A COMPARATIVE EVALUATION OF THE IMMUNOLOGICAL AND BIOLOGICAL TESTS FOR THE DETECTION OF CHORIONIC GONADOTROPHIN IN DIFFERENT CLINICAL CONDITIONS

by

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The antigenic composition of human chorionic gonadotrophin (HCG) and the ability of the anti-HCG antiserum to inhibit the biological activity of the hormone has already been reported by us (Rao and Shahani, 1959, 1961). After establishing the anti-genicity of HCG, attempts were also made to detect the hormone in the urine of pregnant women. The haemagglutination test of Boyden (1951), as modified by Stavitsky (1954) and as standardized in our laboratory (Rao and Sadri, 1960) was used for this purpose. Preliminary work indicated that it should be possible to detect pregnancy by an immunological method (Shahani, 1961).

A double-blind study was also undertaken in collaboration with Dr. B. G. Modi of the Haffkine Institute to detect HCG in the urine samples received by the Institute for the diagnosis of pregnancy by biological test. The preliminary results,

however, were not very encouraging because the results of the immunological test gave only a 60-70% correlation with the biological test. For this as well as several unavoidable reasons we were unable to continue the work as actively as it was necessary to standardize the immunological pregnancy test. Meanwhile several reports were published on the immunological detection of pregnancy, all of which were based on the principle of the haemagglutination inhibition test standardized by Wide & Gemzell (1961, 1962). The reasons for the discrepancy observed by us earlier, have now become evident and are indicated in the results reported in this communication.

We once again got interested in the immunological detection of chorionic gonadotrophin in urine with reference to: (1) assessing the immunological and biological specificity of the chorionic gonadotrophin (CG) and the diagnostic reagents prepared in our laboratory for the detection of the hormone; (2) evaluating the relative specificity and the sensitivity of the immunological and biological methods to detect CG in biological material.

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Received for publication on 1-3-68.

Materials and Methods

Preparation of human chorionic gonadotrophin: Pooled urine from pregnant women was adjusted to pH 5.5 and 4 volumes of absolute alcohol were added to it. The precipitate containing the active material was washed with 50 ml. each of alcohol and ether successively. A 500 mg. quantity of protein (crude hormone material) was obtained from 10 litres of urine.

The crude hormone was further purified on a Sephadex G-200 column (1.5 x 40 cms) using 0.05 M sodium chloride as an eluant. The protein concentration in the fractions was estimated according to the method of Lowry *et al* (1951). Active fractions were pooled. The total yield of the active material was 342 mg. protein. The gonadotrophic activity was estimated by the ventral prostate assay in intact rats (Meyer *et al*, 1961). One mg. of the hormone preparation was found to have a biological potency equivalent to 500 I.U.

Preparation of antiserum to HCG: Antiserum to HCG was prepared by immunizing a sheep with the hormone prepared as already described. A 300 mg. quantity of the hormone was dissolved in 30 ml of saline. Five weekly injections of 0.5 ml in Freund's complete adjuvant were given. The sixth injection consisted only of the solution of the hormones. The sheep was bled 4 days after the last injection. The serum was separated from the clot and sodium azide was added to it as a preservative. The serum was stored at 20° C until required for use. The sheep was reimmunized once more and bled before it was rested. The sera from the vari-

ous bleedings were pooled. The sheep antiserum was diluted 60 times for performing the test.

The haemagglutination inhibition test was employed to detect chorionic gonadotrophin in urine. The procedure employed was as already described by Wide (1962). The HCG used for sensitizing the sheep erythrocytes was obtained as a gift from Dr. M. N. G. Dukes, N. V. Organon, Holland.

The rat ovarian hyperaemia test was employed as a biological method for the detection of HCG. A 5.0 ml quantity of the urine to be tested was injected into 24 to 30 day-old rats and the animals were sacrificed after 24 hours. The test was considered positive if the ovaries of the injected animals became hyperaemic.

The first morning samples of urine referred to our laboratory and to the Haffkine Institute, Bombay, by hospitals and private doctors, as well as some of the 24 hour samples referred to our laboratory, were analysed by both the immunological and biological methods.

Results

Preliminary double blind study: A preliminary double blind study was carried out using the diagnostic reagents prepared as already described. The hundred samples analyzed were those referred to the Haffkine Institute by hospitals as well as by some of the gynaecologists in the city. The results of the immunological test carried out by us were compared with the results of the biological experiments carried out in the Clinical Pathology Department of the Haffkine Institute. It was observed that

the results of the immunological and biological tests tallied in the case of 70 of the samples tested. Of the 30 samples which did not give identical results by the immunological and the biological tests, 19 gave a positive result by the former test and negative by the latter. This result could be explained on the basis that the immunological test is more sensitive. But the most unexpected observation was that the biological test was positive with 11 urine samples while the immunological test was negative. The clinical histories of only 3 out of these 11 cases could be obtained. One of these was a case of threatened abortion, the second had a history of intra-uterine foetal death while the third had 'uterus of 5 months' size and amenorrhoea of 7 months duration'.

Single blind study with the samples referred by the gynaecology department of the Seth G. S. Medical College and the K.E.M. Hospital:

The immunological as well as the biological tests were carried out with 70 urine samples referred during the period. The results of the two tests agreed in 60 of the cases. In those that did not agree there were three cases in which the biological test was positive but the immunological test was negative. Clinical histories were not supplied of all the cases referred and information could not be obtained for all. Two cases out of three had a history of fibroids. The biological test came repeatedly positive and the immunological test negative. The third was a case of tuberculosis of the endometrium.

The results of these preliminary studies indicated the necessity of

carrying out a comparative analysis using these two methods to test urine obtained from different clinical conditions. The results of the analysis are given in Table I.

Strong positive biological test in cases of very early pregnancy with oestroprogestin tablets:

In item (1) in Table I are included the results of analysing two samples of early pregnancy. One of these was of Mrs. R. who was on a schedule of oestroprogestin tablets for the regulation of the menstrual period. A sample of her urine was received on the 14th day after the intake of tablets (she had overlooked taking a tablet on the 18th day of the menstrual cycle) and 8 days after the missed menstrual period. The biological test was strongly positive while the immunological test was negative. A second sample from this case was received 16 days after the missed menstrual period. The immunological test carried out with a concentrated sample of urine gave a strong positive result, the concentration of HCG, however, was low being only 700 I.U. per litre of the urine. Until 20 weeks after the last menstrual period no further samples were available from this case for following the concentration of chorionic gonadotrophin in the urine either by the biological or by the immunological methods. It was of interest to test the assumption that the urine of women given hormonal therapy in early pregnancy would give a strong positive biological test while the more sensitive immunological test was negative or only weakly positive. In order to investigate this possibility Mrs. B. with a history

TABLE I
 Detection of HCG in different clinical conditions by haemagglutination
 inhibition test and rat hyperaemia test

Sr. No.	Different clinical conditions	Total No. of samples tested	Immunological test ±	Biological test ±
1	Normal pregnancy	101
2	Threatened abortions	6
3	Ectopic pregnancy	1
4	Cases with bad obstetric history	38	21	27
5	Suspected pregnancy with hormone therapy	8	4	3*
6	Menopause	4	4
7	Choriocarcinoma	12	12
8	Vesicular mole	9	9
9	Fibroids	16	4**	7
10	Fibroids with pregnancy	2	2
11	Adenomyosis	1	1
12	Tuberculosis of the endometrium.	1	1

*These women were later found to be not pregnant.

**No information could be gathered about the age and the possibility of early pregnancy in these patients.

of 40 days of amenorrhoea, whose urine had given a negative result by the immunological test but a positive result only after concentrating the urine 10-fold, was given two tablets one each on two consecutive days as prescribed by the manufacturer. It was of very great interest to find that the urine collected 2 days following medication with the estroprogestin tablets gave a very strong positive result by the biological test whereas the immunological test was only very weakly positive. At the end of 7 weeks after the missed period the urine sample gave a strong positive result by the immunological test and a weak positive by the biological test. Both the tests were strongly positive at the end of 8 weeks after the last menstrual period.

The second was a case of early pregnancy with a history of 13 weeks of amenorrhoea and 'spotting' who was on a therapy of Proluton Depot. On further inquiry it was found that the patient had a heavy infection of moniliasis. The urine gave good positive results by the biological test. The immunological test was positive only after the urine was concentrated. A second sample at the end of 15 weeks' amenorrhoea and after the infection was treated, gave a strong positive result both by the immunological as well as the biological test.

Discussion

There have been several reviews about the comparative analyses to study the specificity and sensitivity of the various immunological and biological tests to detect HCG in urine and blood. Two of the interesting reviews published recently are

those by Hobson (1966) and Loraine (1966). These workers are of the opinion that the immunological tests are more sensitive and specific for confirming a pregnancy. They, however, feel that the immunological test is not ideal for assessing HCG quantitatively.

Toaff *et al* (1965) are of the opinion that the immunological test and the biological test do not measure identical constituents present in the urine of pregnant women and that the estimation of HCG by the immunological method is cheaper and easier.

It is evident from Table I that the immunological test carried out with reagents employed by us not only gave a better reflection of the clinical picture, but was also helpful for detecting a healthy pregnancy.

In 3 of the 101 cases of normal pregnancy, the immunological test was found to be positive while the biological test was negative. When these 3 cases were followed up, it was observed that both the tests yielded positive results. The titre of HCG as estimated by the immunological test had increased. These results suggest that the immunological test is a more sensitive indicator of pregnancy than the biological test. The results have further revealed that the urine from very early cases of pregnancy, given oestrogen-progesterone combination pills to confirm or rule out pregnancy, or women in their very early pregnancy on medication with Proluton depot seem to give strong positive results by the biological test much earlier than if the women were not on hormonal therapy.

It was of interest to note that in 2

cases of threatened abortion, the immunological test was repeatedly negative while the biological test was positive. The women aborted within 4 to 8 days after the test was carried out. In the remaining 4 cases where the HCG titre was either very low or negligible by the immunological test, the patients aborted within a few days.

Tyler *et al* (1964) observed that false positive reactions were obtained in the immunological test with the urine of women who were receiving high doses of acetylsalicylic acid and related compounds. The immunological test was not positive in the urine of women receiving progestational agents, prednisolone, antibiotics or antispasmodics.

Salzberger and Nelken (1963) using the haemagglutination inhibition test, also obtained positive reactions in the urine of women in their climacteric. Our results also confirm that weak positive results are obtained with urine of women in their early menopause.

In a case of adenomyosis, the immunological test was observed to give a positive reaction. It seems worth finding out whether the invasion of the myometrium by endometrial glands would result in substances being excreted in urine that would act with the anti-HCG antiserum. The results could not be compared with the biological test as the injected animals died. The reason for this is being investigated and will be reported elsewhere.

In the work reported in this communication, positive results were obtained by the immunological as well as the biological tests in a case of

tuberculosis of the endometrium and in 4 cases with uterine fibroids. This could be due to several reasons, such as the protein concentration of urine, the pH of the urine, or even due to high urinary titres of luteinizing hormones, as reported by Venning (1965). An early pregnancy was not ruled out in some of the cases of fibroids.

It would be worth while to investigate the cause of the false positive results, obtained both by the immunological and biological tests, in urine specimens of women with tuberculosis of the endometrium and in certain cases of fibroids.

Studies are also being carried out in two other laboratories in the country to evaluate the specificity and sensitivity of the diagnostic reagents for pregnancy supplied by our laboratory. The results will be presented elsewhere by the workers concerned.

Work is in progress to study, (i) the nature of the substances excreted in the urine of women with fibroids, (ii) results of the diurnal concentration of HCG in urine using the immunological as well as the biological method and (iii) cases of threatened abortions, missed abortions and other gynaecological complications. This work, carried out in collaboration with some of the gynaecologists in Bombay, will be reported elsewhere.

Summary

A comparative analysis of the immunological and biological tests in 199 urine samples obtained from normal and doubtful cases of pregnancy were carried out. The results revealed that the immunological test, using an antiserum to a laboratory HCG

preparation, gave a very reliable correlation with the clinical picture of the patient's condition.

Acknowledgements

It is a pleasure to acknowledge the help and co-operation received from Dr. R. P. Soonawalla, Assistant Honorary, Nowrosji Wadia Maternity Hospital, Parel, Bombay 12, and Dr. C. B. Purandare and Dr. S. D. Chaul from the Department of Obstetrics and Gynaecology, Seth G. S. Medical College and K.E.M. Hospital, in the collection of the clinical material used in the work reported. Our grateful thanks are also due to Dr. S. Dave, who was in charge of the Department of Clinical Pathology, Haffkine Institute, Parel, Bombay 12, for making available some of the urine samples referred to the Haffkine Institute. The preparation of HCG, used in this study, became possible due to the co-operation of women volunteers who supplied the urine between the 10th and 14th weeks of pregnancy.

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