Effects of intrauterine instillation of antiserum to hCG during early pregnancy in mice

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Abstract. Human chorionic gonadotrophin (hCG)-like activity has been reported in mouse and rabbit blastocysts. The presence of this hCG-like activity seems to be essential for nidation of the pre-implantation embryo. Binding sites for hCG were localised on day 4 mouse embryos by immunohistochemical techniques. Presence of hCG-like activity was confirmed by cytotoxicity test. The number of implantation sites was significantly reduced on day 8 of pregnancy. Prior treatment to day 4 mouse embryos with hCG antiserum, for 1 h in utero or in vitro and their subsequent transfer to uteri of synchronised pseudopregnant mice resulted in impaired implantation of embryos, compared to controls treated with normal rabbit serum (NRS). These results suggest that hCG-like activity present on the pre-implantation embryo may have a significant role in implantation of the embryo.

Human chorionic gonadotrophin (hCG)-like activity has been demonstrated in the blastocysts of mice (Wiley 1974; Sengupta et al. 1978) and rabbits (Asch et al. 1979; Varma et al. 1979), employing immunohistochemical techniques and has also been quantitated by radioimmunoassay (Fujimoto et al. 1975) and radioreceptor assays (Haour & Saxena 1974). Furthermore, the fluid from rabbit blastocyst has been reported to cause luteinization of granulosa cells from ovarian follicles of monkey, in vitro (Channing et al. 1978). It is possible that hCG-like activity present on the pre-implantation embryo may be necessary for its nidation. In the present investigation, hCG-like activity was immunoneutralised with antiserum to hCG (As hCG) and the effects of this on implantation of embryo were observed. Furthermore, the presence of binding sites for hCG on the pre-implantation embryo was confirmed, also suggesting a functional role for this substance in blastocyst physiology.

Materials and Methods

Three month old female Swiss mice (body weight 20–22 g, bred in the animal house of the Institute) were used in this study. The animals were kept in an ambient temperature and light controlled room (24°C, 60% humidity and 12 h light/day). Animals showing regular oestrous cycles were allowed to mate with adult males of proved fertility and the presence of vaginal plugs on the following morning confirmed mating, which was considered as day 1 of pregnancy. Pseudopregnancy in mice was induced by cohabitation with vasectomised males.

Antiserum

Antibodies to hCG were raised in male rabbits, using a commercial preparation (Professi 5000 IU/ampoule, Serono, USA). The schedule used was similar to that already reported for ovine LH (Munshi & Rao 1965). Serum samples collected at various intervals were tested for the presence of antibodies by Ouchterlony gel diffusion technique. Sera with high titres of antibodies were pooled and adsorbed with normal human serum. The antibody titre of the pooled sera was 1:80 000 in homologous RIA for hCG (Dattatreayamurti et al. 1975).

Reagents

Antiserum to rabbit gamma globulin (ARGG, dilution 1:45) was raised in sheep at our Institute. Peroxidase antiperoxidase (PAP) was obtained from Polysciences, USA. Tris (hydroxymethyl amino methane): 3.3'-dia-
minobenzidine (DAB) and tryphan blue were purchased from Sigma, USA. Tissue culture medium (MEM199) with Hank’s balanced salt solution was obtained from Samir Laboratory, Bombay.

Presence of binding sites for hCG on day 4 mouse embryo by immunoperoxidase method

The embryos were recovered by flushing the uteri of day 4 pregnant mice with 0.9% saline. The binding sites for hCG were localised immunohistochemically following the technique as described earlier (Purandare et al. 1980). Briefly, the embryos were transferred to the culture medium (MEM 199) containing hCG (10 IU/ml) and incubated at 37°C for 1 h. Subsequently, they were transferred to normal rabbit serum (NRS, n = 25) or As hCG (n = 25, diluted at 1:5 with phosphate buffered saline, PBS, 0.01 M, pH 7.5) and incubated for 1 h at 37°C. Further incubations with ARGG (1:20) and PAP (1:10) were carried out at room temperature for 10 min each. The peroxidase reaction products, obtained after treatment of embryos with DAB-H2O2 (0.1% DAB, containing 0.002% H2O2) were observed under a light microscope.

Localisation of hCG-like antigen by tryphan blue dye exclusion test

Antibodies to hCG were used to detect hCG-like activity on embryo by the tryphan blue dye exclusion test (Hamerlynck & Rümke 1968). Day 4 embryos (n = 25, each) were incubated with 5 µl NRS or As hCG at 37°C for 1 h under humid conditions and then further incubated for 1 h with normal mouse serum (NMS) used as a source of complement. The embryos (n = 25) were also incubated with NMS alone. They were then washed with tissue culture medium and dipped in two drops of tryphan blue (1% solution prepared in 0.9% saline) for 10 min. The embryos were then observed for uptake of the dye under a light microscope.

In utero treatment of embryos with As hCG

To study the effect of in utero administration of As hCG, 31 pregnant mice were allocated to 4 groups. Groups I and II were laparotomised on day 3 of pregnancy and received NRS (5 µl) and As hCG (5 µl), respectively into the right uterine horn. Similarly, groups III and IV were laparotomised on day 4 of pregnancy and received NRS (5 µl) and As hCG (5 µl), respectively into the right uterine horn. The left uterine horn in all the groups was not injected. The NRS treated groups were considered controls to corresponding As hCG treated groups. On day 8 of pregnancy, the animals were autopsied and the number of implantation sites and corpora lutea was noted.

Embryo transfer following in utero exposure to As hCG

In order to determine whether the site of action of As hCG is endometrium and/or embryo, further experiments were planned. Day 4 pregnant female mice were anaesthetised and 5 µl of NRS or As hCG was injected into both uterine horns. Following 1 h of this treatment, the uteri were flushed with tissue culture medium and the embryos were picked up in 5–7 µl of the tissue

Fig. 1.

Photomicrograph of day 4 mouse embryo treated with As hCG. Peroxidase-antiperoxidase reaction visualised by DAB staining on the cells of embryo. × 400.
culture medium, using a microsyringe and transferred to uteri of synchronised day 4 pseudopregnant mice. The number of implantation sites in these recipient mice was noted on day 8 of pseudopregnancy.

Embryo transfer following in vitro exposure to As hCG
Day 4 pregnant mice were autopsied and embryos recovered by flushing the uteri. They were then incubated at 37°C for 1 h under humid conditions along with either 5 µl of NRS or As hCG. Following incubation, they were washed with the medium and transferred to uteri of day 4 pseudopregnant mice. The recipient mice were autopsied and the number of implantation sites counted 4 days after this treatment.

Statistical analysis
Student's unpaired t-test was employed for statistical analysis of the data and the difference was considered significant when P < 0.05.

Results

Immunohistochemical localization of binding sites for hCG on embryo
The dark brown reaction products of PAP were observed on the cells of the As hCG treated day 4 mouse embryos (Fig. 1). In the NRS-treated control embryos (Fig. 2) only a light background staining was observed. In both groups, the zona pellucidae did not take up stain.

Localisation of hCG-like antigen with the tryphan blue dye exclusion test
The embryos when incubated in vitro with the antiserum and complement showed dye positive reaction (Fig. 3) indicating that As hCG cross-reacted with hCG-like antigen on the cells of day 4 embryos. However, on an average only 29% of the embryos stained completely while 48% were partially stained and 23% did not stain. The embryos incubated with NRS and complement or complement alone did not take up thryphan blue dye (Fig. 4).

In utero treatment of embryos with As hCG: effects on implantation
When day 3 or day 4 pregnant mice were treated intraluminally with As hCG, the number of implantation sites in the day 4 treated horn was significantly reduced, compared to those in the NRS injected horn (P < 0.001, Table 1). Correspondingly the number of implantation sites was reduced in the As hCG injected right horn compared to the number of corpora lutea in the right ovary, indicating that implantation was impaired after the treatment.
Fig. 3.
Photomicrograph of day 4 mouse embryo incubated with As hCG + complement showing tryphan blue dye uptake. × 200.

Fig. 4.
Photomicrograph of day 4 mouse embryos incubated with NRS + complement. Note the absence of trypan blue dye uptake. × 200.
Table 1.
Effect of intraluminal injection of As hCG on implantation in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of pregnancy</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Left side</th>
<th>Right side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. corpora lutea in ovary (mean ± SEM)</td>
<td>No. corpora lutea in ovary (mean ± SEM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unjected horn sites (mean ± SEM)</td>
<td>Injected horn sites (mean ± SEM)</td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>NRS</td>
<td>7</td>
<td>5.08 ± 0.50</td>
<td>4.14 ± 0.40</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>As hCG</td>
<td>8</td>
<td>4.12 ± 0.58</td>
<td>4.80 ± 0.39</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>NRS</td>
<td>8</td>
<td>4.96 ± 0.25</td>
<td>4.40 ± 0.08</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>As hCG</td>
<td>8</td>
<td>4.85 ± 0.30</td>
<td>4.30 ± 0.89</td>
</tr>
</tbody>
</table>

*P < 0.001, No. implantation sites in As hCG injected horn vs those in NRS injected horn.
NRS: normal rabbit serum. As hCG: antiserum to hCG.

Embryo transfer to pseudopregnant mice following in utero or in vitro treatment with As hCG
In the As hCG treated in utero group, only 21.5% of the transferred embryos could implant as compared to 67.8% in the NRS treated group (P < 0.001, Table 2). This suggested that the site of action of As hCG is the embryo. This was further confirmed by in vitro incubation of embryos with As hCG, where 38% of the As hCG treated embryos could implant, compared to 63.4% of NRS treated embryos (Table 3).

Discussion
We have demonstrated the presence of hCG-like material on day 4 mouse embryos, and have shown that immunoneutralization of hCG-like material on the pre-implanting embryos inhibited nidation.

The presence of binding sites for hCG on day 4 mouse embryo has been reported (Wiley 1974; Sengupta et al. 1978). Blastocyst has been reported to produce a luteotrophic substance, which maintains corpus luteum function during early pregnancy in rats and rabbits (Zeilmaker & Verhamme 1978; Channing et al. 1978).

Table 2.
Implantation of transferred blastocysts following in utero exposure to As hCG.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. blastocysts transferred (No. recipients)</th>
<th>No. implantation sites (No. recipients)</th>
<th>Per cent implantation (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS</td>
<td>48 (12)</td>
<td>32 (12)</td>
<td>67.8 ± 8.47</td>
</tr>
<tr>
<td>As hCG</td>
<td>35 (12)</td>
<td>9 (12)</td>
<td>21.5 ± 7.10*</td>
</tr>
</tbody>
</table>

*P < 0.001, as compared to NRS treated control.
NRS: normal rabbit serum. As hCG: antiserum to hCG.

Table 3.
Implantation of transferred blastocysts following in vitro exposure to As hCG.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. blastocysts transferred (No. recipients)</th>
<th>No. implantation sites (No. recipients)</th>
<th>Per cent implantation (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS</td>
<td>63 (12)</td>
<td>39 (12)</td>
<td>65.4 ± 5.9</td>
</tr>
<tr>
<td>As hCG</td>
<td>64 (12)</td>
<td>24 (12)</td>
<td>38.0 ± 8.9*</td>
</tr>
</tbody>
</table>

*P < 0.001, as compared to NRS treated control.
NRS: normal rabbit serum. As hCG: antiserum to hCG.

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The trypan blue dye exclusion test was carried out in embryos incubated with As hCG and NMS as complement. It is well known that antibodies bind to the specific antigen and binding of the complement to these antibodies brings about cell lysis, thereby permitting trypan blue dye uptake in the cell (Hamerlynck & Rümkte 1968). Uptake of trypan blue dye by the cells of embryos, after incubation with As hCG and NMS, thus provided evidence of the presence of hCG-like antigen on their surface.

Intraluminal injection of As hCG on day 4 of pregnancy resulted in inhibition of implantation, but less pronounced effects were observed when treatment was given on day 3 of pregnancy. This may be because more pronounced hCG-like activity is present on the embryos on day 4 than that on day 3 (Wiley 1974). Further, slight but not significant reduction in the implantation sites in the uninjected (left) uterine horn was observed when As hCG was injected in the right horn on day 4 of pregnancy. This may be due to diffusion of antisera from the injected (right) horn to the left horn as the mouse uterine horns are known to communicate with each other at the cervical end (Doyle & Margolis 1966).

We did not observe absolute inhibition of implantation by As hCG treatment. There are two possible explanations for this, 1) the dose of antibodies administered may not be sufficient to neutralise the embryonic antigen. We could not verify this, however, as a larger volume of NRS itself (10 and 50 µl) inhibited implantation (50 and 60%, respectively, unpublished data), and 2) it is possible that in addition to hCG-like activity, other pregnancy specific proteins on the embryonic cells may be responsible for implantation, and that these are not neutralised by As hCG.

Sc injection of a 20-fold higher dose (0.1 ml) of As hCG on day 4 post-coitally, failed to inhibit implantation in mice (Nandedkar 1984). This indicated that As hCG in circulation may not be able to neutralise hCG-like material effectively on the implanting embryo. On the other hand intrauterine administration of As hCG inhibits implantation, thus demonstrating a local action of As hCG and not mediated through the maternal circulation. hCG-like activity present in the pre-implantation embryo, may stimulate steroidogenesis or steroid metabolism (Dickmann & Dey 1974; Sengupta et al. 1978; Jones 1983). hCG-like activity in the blastocyst could also be responsible for corpus luteum rescue during early pregnancy (Edwards 1980). Work is under way to explore the mode of action of blastocyst hCG-like activity during implantation.

In conclusion, hCG-like activity is present on the implanting mouse embryo, and is involved in its nidation.

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References


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