Latent effects on \textit{in vitro} development following cytochalasin B treatment of 8-cell mouse embryos

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\textbf{SUMMARY}

Eight-cell mouse embryos when treated with 4-0/\mu g/ml cytochalasin B (CB) \textit{in vitro} undergo a reversible developmental arrest. Upon rinsing of embryos and subsequent culture in control medium, normal morphogenetic processes such as compaction of 8-cell embryos, cavitation, and post-blastocyst attachment and outgrowth are restored. However, the effects of CB on mouse embryos are not completely reversible; latent post-blastocyst defects become increasingly more prevalent as CB treatment duration increases.

The present study was conducted to quantitatively determine latent effects of CB on post-blastocyst embryos by comparing their ability to attach and to sustain the growth and differentiation of ICM and trophoblast tissues. Groups of 8-cell embryos were cultured in Brinster's BMOC-3 medium containing 4-0/\mu g/ml cytochalasin B for 6, 12, 18, and 24 h. Following treatment, embryos were rinsed and cultured until 190 h post coitum (h.p.c.) in Eagle's MEM/10% fetal calf serum modified to contain optimal levels of essential amino acids. Blastocysts generally attached to the surface of the plastic substratum by 120 h.p.c. At selected time periods after attachment (130, 160, and 190 h.p.c.), embryos were scored for outgrowth size, ICM size, extent of peripheral hyaloplasmic fan, and number of trophoblast nuclei per outgrowth. Analyses of variance (ANOVAs) were conducted for each of the four parameters listed above. Rates of attachment were analyzed by $\chi^2$ test.

Results show that the treatments affect ($P < 0.01$) embryo attachment, number of trophoblast nuclei per outgrowth, hyaloplasmic fan production, and ICM growth in a duration-dependent manner. Interestingly, since treatment effects on outgrowth areas are nonsignificant apparently CB does not significantly change total outgrowth area. But CB treatment does cause abnormal fan production and decreased trophoblast nuclei numbers. However, trophoblast cells are apparently more resistant than ICM to CB as is evident by the high incidence of trophoblast outgrowths devoid of ICM.

CB (4-0/\mu g/ml) treatments at 8-cell stages for relatively short durations (6 and 12 h) induce latent effects on post-blastocyst embryos. Finally, there exists a definite 4-0/\mu g/ml CB duration response over the 68–190 h.p.c. observation interval.

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INTRODUCTION

Treatment of preimplantation mouse embryos with 4–10 μg/ml CB results in: (1) production of tetraploids and 2n/4n mosaics by suppression of the second cleavage division (Snow, 1973; Tarkowski, Witkowska & Opas, 1977), (2) a reverse compaction of compacted 8-cell embryos (Ducibella & Anderson, 1975), and (3) failure of embryos to cavitate and to produce ICM and trophoblast cell populations (Granholm & Brenner, 1976a). Interestingly, the overall reversibility of the effects of CB on long-term development seems to be extraordinary as judged by the birth of transferred tetraploid young (Snow, 1975). Indeed, even after a 24 h pulse in 4.0 μg/ml CB, 46/48 or 96% of treated 8-cell embryos cavitated to form blastocysts (Granholm & Brenner, 1976a).

Upon examination, however, a number of CB-induced developmental defects have been discovered and described. After transferring CB-treated 2-cell mouse embryos to recipients, development was clearly abnormal by the eighth day of pregnancy (Tarkowski et al. 1977). After similar treatments Snow (1976) reported that only 19.4% (6/31) of tetraploid blastocysts in early implantation sites possessed both ICM and trophoblast cell derivatives; where ICM derivatives were present, their development was generally abnormal. However such abnormalities were apparently consistent with live birth, since 0.5% (4/846) similarly treated 2-cell embryos when transferred developed to term as tetraploids (Snow, 1975).

It is, therefore, of interest to determine the extent to which CB-treated preimplantation embryos can recover and undergo normal post-implantation morphogenesis. Moreover, CB may produce selective and predictable inhibitory effects on trophoblast or ICM derivatives as reported for certain metabolic inhibitors (Epstein, 1975; Rowinski, Solter & Koprowski, 1975).

The present study was conducted to quantitatively determine the latent effects of varying pulses of CB on early post-implantation mouse embryos by comparing their ability to hatch, to attach, and to sustain the growth and differentiation of ICM and trophoblast tissue. In contrast to previous studies, CB treatment began at the 8-cell stage, thus suppressing the fourth cleavage division.

MATERIAL AND METHODS

Eight-cell embryos were recovered from superovulated virgin ICR females, pooled, and randomly assigned to the following treatment or control groups at 68 h.p.c.:

1) Brinster’s BMOC-3 media with 4.0 μl/ml dimethylsulphoxide (DMSO) control group.

2) 6, 12, 18, or 24 h 4.0 μg/ml CB treatment groups.

Cytochalsin B was dissolved in DMSO and added to Brinster’s BMOC-3 medium to make a final concentration of 4.0 μg/ml CB. The dosage of 4.0
In vitro development of mouse embryos was selected on the basis of previous CB dose response data; 4.0 μg/ml was the lowest concentration of the drug that completely inhibited the morula-to-blastocyst transformation between 68–92 h.p.c. (Granholm & Brenner, 1976a). Embryos were cultured in 2.0 ml of media in Falcon dishes (No. 3001) at 37 °C, gassed with 5% CO₂ in air.

At the end of the period allotted for the cytochalasin B pulse (6, 12, 18, or 24 h), the treatment group was rinsed three times in Brinster's medium to remove CB, and subsequently cultured in Brinster's BMOC-3. DMSO control embryos were rinsed after 24 h. At 92 h.p.c. all treatment groups and control groups were cultured in Eagle's MEM/10% fetal calf serum modified to contain optimal levels of essential amino acids to allow for post-blastocyst development (Spindle & Pederson, 1973). Fetal calf serum (Gibco) was pretested for its ability to support growth and differentiation of early mouse embryos in vitro.

In order to facilitate location of blastocysts after attachment, blastocysts were placed in 12 mm diameter x 7 mm high glass rings attached with silicone grease (Dow) to the bottom of Falcon dishes (No. 3001) containing medium both within and outside of the ring. Medium was added until approximately 6 mm of the ring was submerged (2.0–3.0 ml). After attachment at 120–130 h.p.c., the rings were removed and all groups were scored for the following parameters: (1) size of outgrowth, (2) size of ICM, (3) extent of hyaloplasmic fan per outgrowth, and (4) number of trophoblast cell nuclei per square millimeter of outgrowth area. These parameters were scored by taking photographs at 50× magnification during each time period. A transparent calibrated grid containing evenly spaced points was superimposed over individual contact prints; areas were determined by counting points (n) falling on or within the perimeter of individual outgrowths, fans, and ICMs. This method measures absolute area; absolute area = nd², where d is the distance between adjacent points (Halley, 1964). Nuclei of trophoblast cells of outgrowths were counted from contact prints of outgrowths. Number of nuclei and fan regions were adjusted to outgrowth size so that these parameters are independent of area of outgrowth, which can vary considerably. Counts of nuclei were expressed as number of nuclei per millimeter squared of outgrowth, and fan regions were expressed as proportion of fan region relative to outgrowth area.

A factorial experimental design was applied to the data. Groups of 12 embryos were treated with cytochalasin B for evenly spaced pulse durations (Factor C). Each treatment and control was replicated six times (Factor B). To accomplish this replication each block (replication) consisted of 60 semicompacted or compacted 8-cell embryos obtained at a given recovery. Scoring of parameters was done over three equally spaced time periods (Factor A). The data were analyzed on an IBM computer and the results presented in an analysis of variance table (Table 2). In all ANOVAs, replications are random while everything else is fixed. Response curves were performed on significant main effects.
by curvilinear regression techniques. An IBM program was used to fit the data to polynomials of increasing degrees, and the results were presented graphically (Fig. 2).

RESULTS

Preimplantation development during and following CB treatment

At a concentration of 4.0 µg/ml CB blocks cytokinesis, and embryos retain the cell number they possessed when first subjected to the drug (Granholm & Brenner, 1976a). Cleavage-stage embryos when treated with 4.0 µg/ml CB in BMOC-3 at 68 h.p.c. remained as cleavage-stage embryos throughout the treatment duration, and individual blastomeres of 8-cell uncompacted embryos retained their rounded appearance. Blastomeres of compacted 8-cell embryos underwent a reverse compaction process (Ducibella & Anderson, 1975) characterized by apparent changes in embryo transparency and blastomere shape. CB-treated embryos become more transparent (and less translucent) than untreated controls. Blastomeres become more spherical with obvious modifications in the extent of cell-to-cell contacts.

A high percentage of embryos were able to recover from CB treatment after rinsing with culture medium. After 24 h of 4.0 µg/ml CB treatments, 61/71 or 85.9% formed definitive blastocysts within 12 h of removal of CB. However, abnormalities were seen in many of these recovered embryos including reticulation or partitioning of the blastocoel, thickened trophoblast epithelia, formation of small and contracted blastocysts, accumulations of small ICM foci (ectopic ICMs) distributed in various positions around blastocoelic spaces, and oddly shaped blastocysts possessing numerous surface bulges.

Effects of CB on attachment

Pulse durations of CB were tested for effects upon attachment. There was no significant difference between replications, and data were pooled. Combined results were analyzed with a $\chi^2$ test for significant differences of attachment among groups at 130 h.p.c. ($\chi^2 = 83.10$, $P < 0.01$). The analysis indicated no significant difference between the control and 6 h CB treatment ($\chi^2 = 0.012$, n.s.). The average of the control and the 6 h treatment was found to be significantly different from 12 h CB treatment ($\chi^2 = 3.87$, $P < 0.05$). Similarly, the average of control, 6 h, and 12 h treatments was significantly different ($P < 0.01$) from 18 h treatment, as was the 24 h treatment from the average of the other four groups. Thus, it can be concluded that CB pulses for greater than 6 h have deleterious effects on attachment. An examination of the results (Table 1) indicates that the number of embryos which attached by 130 h.p.c. decreased with increasing pulse duration.
In vitro development of mouse embryos

Table 1. Attachment of blastocysts at 130 h.p.c.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Attached</th>
<th>Non-attached</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59</td>
<td>11</td>
<td>70</td>
</tr>
<tr>
<td>6 h</td>
<td>55</td>
<td>14</td>
<td>69</td>
</tr>
<tr>
<td>12 h</td>
<td>46</td>
<td>22</td>
<td>68</td>
</tr>
<tr>
<td>18 h</td>
<td>25</td>
<td>44</td>
<td>69</td>
</tr>
<tr>
<td>24 h</td>
<td>17</td>
<td>54</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>145</td>
<td>347</td>
</tr>
</tbody>
</table>

Latent CB effects on post-implantation development

Four parameters of post-implantation development were analyzed at 130, 160, and 190 h.p.c. intervals. Analyses of variance (ANOVAs) for each of the four parameters are presented in Table 2. Significant effects ($P < 0.01$) are indicated by the double asterisks. For outgrowth area, ANOVA shows that culture time and replication are significant. Examination of the means for culture time indicated that outgrowth size increased consistently over the three time periods.

For trophoblast nuclei per outgrowth (mm²), ANOVA shows that both time and treatment are significant. To determine if the response changes systematically as pulse duration increases, data were analyzed by curvilinear regression. A linear relationship (Fig. 2a) was found to exist with number of nuclei decreasing with increased pulse duration. The equation of the duration response line is $\hat{Y} = 483.4 - 75.5(X)$, where $\hat{Y}$ is the expected number of nuclei per outgrowth (mm²) and $X$ is the treatment number (1, 2, ...5) where 1 = control, 2 = 6 h CB pulse, 3 = 12 h CB pulse, 4 = 18 h CB pulse, and 5 = 24 h CB pulse. The standard error of estimate is 140.7.

Clear hyaloplasmic regions of outgrowing trophoblast cells are often observed (Fig. 1d,f). Time-lapse films have shown that hyaloplasmic fans exhibit ruffling membrane activity; CB produced an immediate cessation of ruffling activity (Granholm & Brenner, 1976a). Moreover, other studies revealed that some CB treated embryos possessed abnormally extensive peripheral hyaloplasmic fan regions (Granholm & Brenner, 1976b). It was, therefore, of interest to measure hyaloplasmic fan regions after pulsing with CB. Proportions of clear fan area relative to total outgrowth area were analyzed (Table 2). Treatments were found to be highly significant. A response curve was performed on the data (Fig. 2b). The equation of the fitted response is $\hat{Y} = 0.1855 + 0.0600(X)$. The standard error of estimate is 0.1330. It is seen that the response is linear, with fan areas increasing with pulse duration.

Areas of ICMs were measured on attached embryos and analyzed (Table 2). ANOVA shows that the main effects for treatments and time are significant. An examination of means for time in culture indicated a trend of decreasing ICM area over the three time periods. The fitted response function for treatment
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>A = time</th>
<th>B = replication</th>
<th>C = treatment</th>
<th>AxB</th>
<th>AxC</th>
<th>BxC</th>
<th>AxBxC</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees of freedom</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>20</td>
<td>40</td>
<td>387</td>
</tr>
<tr>
<td>Mean square</td>
<td>0.10849</td>
<td>0.0023**</td>
<td>0.00063**</td>
<td>0.00046**</td>
<td>0.00088**</td>
<td>0.00189**</td>
<td>0.00073</td>
<td></td>
</tr>
<tr>
<td>Trophoblast nuclei per outgrowth (mm²)</td>
<td>223.2763**</td>
<td>177.517</td>
<td>92.825.3**</td>
<td>14.112.1</td>
<td>92.4825**</td>
<td>21.328.4</td>
<td>169.187.4</td>
<td></td>
</tr>
<tr>
<td>Outgrowth area</td>
<td>0.0001**</td>
<td>0.000223**</td>
<td>0.000062**</td>
<td>0.000046**</td>
<td>0.000088**</td>
<td>0.000180**</td>
<td>0.000054</td>
<td></td>
</tr>
<tr>
<td>Hyaloplasmic fan area per outgrowth</td>
<td>0.00018**</td>
<td>0.0000299</td>
<td>0.000032**</td>
<td>0.000013</td>
<td>0.000026**</td>
<td>0.000018**</td>
<td>0.0000026</td>
<td></td>
</tr>
<tr>
<td>ICM area</td>
<td>0.0001188**</td>
<td>0.0000299</td>
<td>0.000032**</td>
<td>0.000013</td>
<td>0.000026**</td>
<td>0.000018**</td>
<td>0.0000026</td>
<td></td>
</tr>
</tbody>
</table>

** P < 0.01.
† Proportion of clear fan area relative to outgrowth area.
Fig. 1. Latent effects of CB on outgrowths of mouse embryos in vitro. (a, c, e) DMSO control embryos at 130, 160, and 190 h.p.c., respectively. For these embryos, outgrowth areas increased, number of trophoblast nuclei per outgrowth increased, and ICM areas decreased. Note the differentiation of ICM in (e) to form inner ectoderm and outer endoderm. Magnifications are ×209, ×190, and ×110, respectively. (b) Embryo at 130 h.p.c. following 12 h 4-0 μg/ml CB treatment from 68 to 80 h.p.c. ×293. (d, f) Twelve- and eighteen-hour 4-0 μg/ml CB-treated embryos showing extensive peripheral hyaloplasmic regions (arrowheads), fewer trophoblast nuclei, and few, if any, ICM cells. Outgrowths in (d) (×183) and (f) (×146) were photographed at 160 and 190 h.p.c., respectively.
Fig. 2. Duration–response lines of 4.0 μg/ml CB on (a) number of trophoblast nuclei per outgrowth (mm²), (b) proportion of fan area per outgrowth area, and (c) size of ICM (mm² x 10⁻³). Response curves were generated by curvilinear regression techniques. For each parameter measured, the linear equation was significant and plotted graphically. The equation of the duration–response line and the standard error of estimate are given with each graph. In each instance, $\hat{Y}$ is the expected number and $X$ is the treatment number (1, 2, ..., 5), where 1 = control, 2 = 6 h CB pulse, 3 = 12 h CB pulse, 4 = 18 h CB pulse, and 5 = 24 h CB pulse.
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Effects is presented graphically in Fig. 2(c). The equation of the curve is $\hat{Y} = 0.00868 - 0.00115(X)$. The standard error of estimate is 0.0055. The response is linear; ICM area decreased with increasing CB pulse duration.

**DISCUSSION**

We previously reported that 4.0 µg/ml CB induces developmental arrest of 8-cell embryos over a 6–24 h treatment period and that many arrested embryos subsequently transform into apparently normal blastocysts after rinsing and culture in control medium (Granholm & Brenner, 1976). Following recovery from CB, blastocysts possess fewer than normal cells, partitioned blastocoels, thickened trophoblast epithelia, ectopic ICMs, and abnormal trophoblast bulges. The occurrence of these morphological abnormalities suggests that CB prevents the orderly differentiation and segregation of ICM and trophoblast components possibly by interfering in specific ways with morphogenetic cell movements (Miranda, Godman & Tanenbaum, 1974; Perry & Snow, 1974).

In addition to preimplantation effects, data presented in this paper show that treatment of 68 h.p.c. embryos with 4.0 µg/ml CB for 6, 12, 18, and 24 h also impairs the subsequent attachment of blastocysts to substrata and the development of ICM and trophoblast derivatives. Percentages of attachment varied inversely with the duration of CB treatment (Table 1). CB-induced effects on attachment could possibly be caused by preventing hatching from the zona pellucida, by interfering with microfilaments essential for the initial adhesion of trophoblast processes to the substratum, by decreasing the number of ICM cells below a critical threshold level thereby secondarily inhibiting trophoblast cell attachment (Ansell & Snow, 1975), or by polyploidy and cell death induced by CB treatment. Snow (1976) reports that 37/63 (58.7%) CB-induced tetraploid embryos produced outgrowths in vitro as compared to 29/30 (96.7%) diploids. Although further experiments are necessary to identify the mode of action of CB on blastocyst hatching or attachment, our results definitely show a duration-response relationship (Table 1); 84.3% of the DMSO controls versus 79.9, 67.6, 36.2, and 23.9% of the 6, 12, 18, and 24 h CB-treated embryos attached.

Regarding post-implantation development of DMSO control embryos to 190 h.p.c. (Fig. 1a, c, e; Table 2), outgrowth areas increased ($P < 0.01$), number of trophoblast nuclei per outgrowth increased ($P < 0.01$), and ICM areas decreased ($P < 0.01$). Reasons for ICM area decreases over time may be adverse culture conditions or perhaps an expression of the reorganization of ICM cells from a flattened spread configuration to a more consolidated cylindrical arrangement which when viewed through a microscope, photographed, and scored would have less of an areal extent than a flattened conformation.

Following CB pulses, treatment effects are seen (Fig. 1b, d, f; Table 2) on the number of trophoblast nuclei, hyaloplasmic fan elaboration, and ICM
area in a duration dependent manner. It thus appears that latent effects of CB on post-blastocyst development can be predictably determined by the timing and duration of treatment.

In the present study, CB treatments were not found to change total outgrowth areas. However, the number of nuclei per outgrowth decreased with increasing pulse duration, while the areas of hyaloplasmic fan regions increased. These observations suggest that although total areas of outgrowths were not significantly changed by CB treatments, CB was harmful to primary trophoblast outgrowth in vitro. Interestingly, the production of abnormal fan regions has not been observed with other inhibitors, and this defect appears to be specific for CB treatments. Granholm & Brenner (1976a) have suggested that CB may interact with microfilaments so as to alter subsequent development of hyaloplasmic regions.

The effects of CB on ICM areas indicate a differential survival of ICM versus trophoblast. Although CB treatment does produce latent effects on trophoblast outgrowth as is evident by abnormal fan production and by reduced trophoblast nuclei numbers, trophoblast cells are apparently more resistant to CB treatment as is evident by the high incidence of trophoblast outgrowths devoid of ICM. A similar observation was made by Snow (1975). An in vitro study showed fewer tetraploid blastocysts produced by treating 2-cell mouse embryos with 12 h 10-0 μg/ml CB pulses than control blastocysts outgrew; also, all control (diploid) outgrowths possessed ICMs, but in 19/31 of the tetraploid outgrowths ICMs were absent. In utero, 67% of transferred tetraploid blastocysts in early implantation sites lacked ICM cells. These results suggest that CB interferes with formation of ICM in the blastocyst, possibly by interfering with microfilament-mediated cell movements or by causing selective cell death. Moreover, trophoblast growth and differentiation may have been deleteriously affected by lack of ICM in addition to CB treatment effects. Our observations show both ICM areas and trophoblast cells numbers decreased with increasing pulse duration.

The latent effects of CB on post-blastocyst development may be partially determined by the timing and duration of CB treatments. For example, tetraploid blastocysts produced by treating 2-cell mouse embryos with 10-0 μg/ml CB over the 40-52 h.p.c. interval and subsequently transferred to pseudopregnant recipients can result in the production of viable but diminutive offspring in a small percentage of cases, indicating that CB-induced tetraploidy is compatible with morphogenesis and survival until parturition (Snow, 1975). However, it was shown that the majority of blastocysts develop in utero only as trophoblast giant cells and that few show evidence of an ICM. Where ICM derivatives were present, development was not necessarily normal and a high incidence of embryonic mortality was apparent through gestation. In contrast, developmental effects of tetraploidy induced by 3-8½ h 10-0 μg/ml CB treatment were not observed until the eighth day of pregnancy (Tarkowski et al. 1977).
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The first symptom of tetraploidy was retardation of the development of the embryonic part of the egg cylinder, but the main problem was inadequate production of mesoderm. None of the implanted tetraploid blastocysts were able to survive until birth. It may be that the shorter duration of CB treatment may result in less harmful effects, although differences in methodology and mice strains could have contributed to the different developmental potential of CB-treated mouse embryos.

We have found that post-blastocyst effects of CB treatment increased from 6 to 24 h. Effects of 12 h or greater treatments of preimplantation embryos indicate that CB may in fact be more harmful to cells contributing to the developing embryo than to those cells predestined to become fetal membranes. CB-induced polyploidy may reduce proliferation and viability of cells, and would, therefore, be expected to adversely affect development of the embryo proper rather than affect cells of extra-embryonic membranes some being derived from primary trophoblast, which is polyploid by nature. In this same respect, it is interesting that in 2n/4n mosaics, 4n cells are eliminated from embryos but not from fetal membranes (Tarkowski et al. 1977).

In summary, latent effects of 4-0 μg/ml CB pulses included inhibition of ICM growth; treatment effects were also seen to reduce trophoblast nuclei and to stimulate abnormal fan production of trophoblast outgrowths. However, trophoblast cells appear to be more resistant than ICM cells to CB treatment as is evident by the high incidence of trophoblast monolayers devoid of ICM and by the decrease in ICM areas with increasing pulse duration.

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