Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos

By A. K. TARKOWSKI, A. WITKOWSKA AND J. OPAS

From the Department of Embryology, Institute of Zoology, University of Warsaw

SUMMARY

By subjecting F₁ (CBA × C57/BL) × A eggs at the time of 2nd cleavage to 10 μg/ml of cytochalasin B (CB), tetraploidy was produced in 52% of 2-cell eggs and 35% of 3-cell eggs. 2n/4n mosaic embryos were produced from 2-, 3- and 4-cell eggs and amounted to 20% of all treated eggs. 80% of tetraploid embryos developed in vitro into regular blastocysts with half the cell number of control diploids. The effectiveness of CB in producing tetraploid embryos is limited by the asynchrony of 2nd cleavage, both between eggs and between sister blastomeres. Two-cell presumed tetraploids were transplanted to recipients and examined between the 6th and 11th day of pregnancy. Up to 6½ days development is normal and most embryos form egg-cylinders. At 7½ days the embryonic part of the cylinders is underdeveloped and in later development fails to form an embryo. Development of foetal membranes is much less affected and in the most successfully developing egg-cylinders their formation can be fully accomplished. Failure of embryonic development appears to be due to subnormal activity of the primitive streak, resulting in shortage of mesoderm. Postimplantation development of 2n/4n mosaics was normal. While in embryos tetraploid cells were either absent or in very low proportion (below 4%), their contribution to the foetal membranes amounted in some cases to up to 50%. Elimination of tetraploid cells from mosaic embryos suggests that they have a lower proliferation rate than diploid cells.

INTRODUCTION

Spontaneous tetraploidy is a relatively rare chromosomal aberration in mammals. In the mouse, preimplantation tetraploid embryos have been identified by Beatty and Fischberg (summarized by Beatty, 1957), but their survival beyond implantation has not so far been described. In Man, tetraploid embryos can implant but are aborted in early pregnancy, the only exception being a liveborn infant described recently by Golbus, Bachman, Wiltse & Hall (1976). Abortuses consist of foetal membranes only and do not contain any recognizable embryonic structures (Carr, 1971; Hamerton, 1971).

First attempts to induce tetraploid development in the mouse were carried out by Beatty and Fischberg (1952, suppression of first cleavage by heat shock applied to the oviduct) and by Edwards (1958a and b, injection of colchicine at the time of fertilization or first cleavage) but were limited to the preimplantation period. Recently tetraploid mouse blastocysts were produced by virus-
assisted fusion of diploid pronucleate eggs or blastomeres from 2-cell embryos (Graham, 1971; Tarkowski, Wróblewska and Bałakier, unpublished). Graham transplanted 4n blastocysts to recipient females and observed implantation sites that contained only trophoblastic giant cells.

A few years ago Dr M. H. L. Snow in Edinburgh and our group started experiments aimed at inducing tetraploidy in the mouse with the help of a mould-metabolite, cytochalasin B. The technique worked out independently in these two laboratories proved to be superior to the methods previously employed and yields tetraploid embryos routinely and on a large scale. The results obtained by Snow have already been published in a series of reports (Snow, 1973, 1975, 1976; Perry & Snow, 1975).

The utilization of cytochalasin B (CB) as a tool to produce polyploidy exploits the fact that the drug suppresses cytokinesis without interfering with karyokinesis. Since 1967 when Carter described this biological effect of cytochalasins, CB has been extensively employed in studies on the mechanism of cell division in various types of cells, including oocytes and cleaving eggs. CB is known to suppress maturation divisions in oocytes of the snail Spisula (Longo, 1972) and of the mouse (Niemierko, 1975; Bałakier and Tarkowski, 1975; Niemierko and Komar, 1976) and to block cleavage divisions in sea urchins (Schroeder, 1968, 1972), amphibians (see Luchtel, Bluemink & de Laat, 1976 for references to earlier publications) and in the mouse (Snow, loc. cit., and this paper). However, out of all these studies only those carried out on the mouse are concerned with the effects of CB-induced polyploidy on embryonic development.

In the present study we describe the induction of tetraploidy and of diploid/tetraploid mosaicism in the mouse by subjecting eggs at the time of second cleavage to CB and we also depict the developmental effects of these aberrations. Contrary to the studies by Snow (loc. cit.), who observed in a certain number of cases development of tetraploid foetuses up to birth, our tetraploid embryos consistently showed a definite developmental anomaly. This anomaly begins to manifest about the 8th day of pregnancy and inhibits or severely interferes with the formation of the embryo but not of the foetal membranes.

MATERIALS AND METHODS

Animals

Donors: F_1 (CBA × C57BL/10) females, 2–4 month old, induced to ovulate (5 i.u. each of PMSG and HCG given 42–48 h apart) and mated with A males.

Recipients: Spontaneously ovulating Swiss albino females mated with vasectomized males of A strain.

Treatment of eggs in vitro

Eggs were collected 47–49½ h post HCG. Presence of 3- and 4-cell eggs (about 20 % of all eggs recovered) indicated that the 2nd cleavage had already begun.
Eggs from each female were treated separately as one batch. After being separated from 3- and 4-cell embryos, 2-cell embryos were divided into groups and subjected to one of the following treatments:

1. Culture medium (CM) – control I.
2. Culture medium with 1% dimethyl sulfoxide (DMSO) – control II.
3. Culture medium with DMSO and CB (CB) – experimental.

Cytochalasin B (Imperial Chemicals Ltd) was dissolved in DMSO (Carter, 1967) and added to culture medium (Whitten, 1971) to make the concentrations of CB and DMSO 10 μg/ml and 1% respectively. Eggs were cultured in drops of media under liquid paraffin in siliconized glass dishes at 37 °C, gassed with 5% CO₂ in air.

Two-cell control embryos were examined every 1–2 h, and the sister experimental embryos were removed from CB after all, or at least the overwhelming majority of control eggs reached the 4-cell stage. The duration of treatment with CB was thus regulated individually for each batch and varied between 3 and 8½ h (average time for all experiments – 4½ h). The experimental embryos were washed thoroughly and placed in CM. After 2–4 h they were carefully examined. The regular 2-cell ones were separated from those which resumed cleavage or showed symptoms of fragmentation (see Results), and either transplanted or left in culture for 48–55 h.

All 3-cell embryos and a number of 4-cell embryos were treated with CB in a similar manner and those which reverted to the 2- or 3-cell stage were either cultured to the blastocyst stage or transplanted on the same day as presumed tetraploids or diploid/tetraploid mosaics.

The following groups of embryos served as diploid controls in transplantation experiments:

1. Eggs subjected to CB at the 4-cell stage, which remained 4-cell after treatment,
2. 2-cell eggs which underwent 2nd cleavage in DMSO, and
3. 2-cell eggs which became 4-cell in CM soon after CB treatment.

In an additional small series of experiments eggs were left in culture overnight (14–20 h) to undergo 3rd cleavage and then the 2n and 4n embryos were segregated before transplantation on the basis of the number of blastomeres: 8-cell – diploids, 4-cell – presumed tetraploids.

In order to determine the state of the nuclear apparatus during and shortly after CB treatment a number of eggs were fixed and examined in permanent haematoxylin preparations (Tarkowski, 1971). At the end of culture the stage of development was defined and air-dried preparations were made to determine the number of cells and ploidy (Tarkowski, 1966). In some cultures colchicine (ca. 0.2 μg/ml) was added a few hours before making preparations.
Transplantation

Embryos were transferred to the oviduct on the 1st day of pseudopregnancy or to the uterus on the 3rd day (day of vaginal plug = 1st day) under Avertin anaesthesia (0.01 ml of 2% solution/g body weight).

Examination of implanted embryos

Tetraploid embryos. The recipient females were killed between the 6th and 11th day of pregnancy. Implantations were either processed for histology (Heidenhein fixative (‘Suza’), 6-μm sections stained with haematoxylin and eosin) or torn open under dissecting microscope (9-11th days only). From the dissected egg-cylinders chromosomal preparations were made using the technique of Evans, Burtenshaw & Ford (1972).

Diploid/tetraploid mosaic embryos. Conceptuses were dissected from the uteri on the 9th, 10th and 13th day of pregnancy and chromosomal preparations were made separately from the embryos and the foetal membranes.

RESULTS

Reaction of eggs to CB

2-cell eggs

Eggs were usually first observed after 1 h in culture and we therefore do not have detailed observations on the ‘blebbing’ phenomenon which according to Snow (1973) and Perry and Snow (1975) occurs in some eggs immediately after they are subjected to CB. After this short-lasting reaction the eggs resume regular shape and smooth surface and retain this appearance as long as they stay in CB (Fig. 1). When CB is replaced by CB-free medium, the blastomeres in many eggs become wrinkled and irregular and show symptoms of fragmentation (Fig. 2). Some of these eggs and also some of those which remained smooth resume cleavage (in one or both blastomeres) but in the others the surface of blastomeres steadily ‘smooths out’ and after a few hours they regain a regular

<table>
<thead>
<tr>
<th>Stage of eggs subjected to CB</th>
<th>No. of eggs</th>
<th>2-cell</th>
<th>3-cell</th>
<th>4-cell</th>
<th>Pseudofragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>400</td>
<td>207 (51.8)</td>
<td>108 (27.0)</td>
<td></td>
<td>85 (21.2)*</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>27 (35.5)</td>
<td>49 (64.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>2 (2.1)</td>
<td>21 (21.9)</td>
<td>73 (76.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>572</td>
<td>236 (41.3)</td>
<td>70 (40.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Out of 37 eggs cultured overnight there were 20 8-cell and 17 4-cell.
† Number and percentage refer only to those 3-cell mosaic eggs which were produced with CB from 3- and 4-cell eggs.
shape. In view of their transient character these surface changes should be called pseudofragmentation rather than fragmentation. When pseudofragmenting eggs are left in culture overnight they develop into regular 8-cell embryos (diploid) and 4-cell embryos (presumably tetraploid) without cytoplasmic fragments in the perivitelline space. Because some eggs resume cleavage after CB treatment and because characterization of pseudofragmenting eggs is impossible, the selection of 4n and 2n/4n embryos should be carried out a few hours after placing the eggs in CB-free culture medium. The figures shown in Table 1 are based on examination of eggs after 2-4 h and present a good approximation to the definite effects of treatment with CB.

Among eggs which resumed cleavage or were pseudofragmenting, some eventually became 3-cell (presumed diploid/tetraploid mosaics). In a group of 171 eggs (a portion of 400 eggs shown in Table 1) whose fate was closely followed and which were precisely classified after a longer period of time there were 19 such eggs (11.1%).

In cleaving mouse eggs CB has no effect on karyokinesis and on growth of the resulting sister nuclei (Fig. 3). The blastomeres in which cytokinesis is effectively suppressed remain binucleate until the next cleavage, when the two nuclei contribute chromosomes to a single tetraploid metaphase plate (Fig. 7).

3- and 4-cell eggs

A substantial number of these eggs – most likely those in which blastomeres had just undergone cytokinesis – reverted in CB to the 2- or 3-cell stage, giving rise to presumed tetraploids and 2n/4n mosaics respectively (Table 1, Fig. 4). Upon removal from CB, the 3- and 4-cell eggs behaved slightly differently from the 2-cell eggs: wrinkling of the cell surface was less intense and lasted for a shorter time and resumption of cleavage was observed only sporadically.

Preimplantation development of tetraploid and mosaic embryos

Preimplantation development of 2-cell eggs subjected to various treatments is summarized in Table 2. Table 3 compares development of tetraploid, mosaic and diploid embryos produced from 2-, 3- and 4-cell eggs.

Two-cell eggs in which the 2nd cleavage was effectively suppressed in both blastomeres (potential tetraploids) developed into blastocysts in about 80% as compared to 90% in the two control series. Both experimental and control blastocysts were regular and possessed an inner cell mass (Fig. 6), but differed significantly in cell number. The very low cell number in tetraploid embryos – 16.7 on average in the main series of experiments, and 19.3 in the whole available material – can be accounted for by the omission of one cleavage, together with the harmful effect of DMSO. This effect is evident from the comparison of the mean numbers of cells in DMSO and CM control series (Table 2).

The standard procedure followed in the majority of experiments (examination
Table 2. Development in vitro of experimental and control 2-cell eggs (combined results of 4 experiments carried out simultaneously; treatment with CB varied between 4\frac{1}{2} and 8\frac{1}{2} h)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Presumed ploidy</th>
<th>Total no. of embryos</th>
<th>No. and stage of embryos</th>
<th>Mean no. of cells</th>
<th>Mean no. of cells in control embryos (CM)</th>
<th>Estimated ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>2n</td>
<td>37</td>
<td>M – morulae</td>
<td>40·7</td>
<td>–</td>
<td>2n</td>
</tr>
<tr>
<td>DMSO</td>
<td>2n</td>
<td>22</td>
<td>B – blastocysts</td>
<td>34·3</td>
<td>0·84</td>
<td>2n</td>
</tr>
<tr>
<td>CB</td>
<td>4n*</td>
<td>25</td>
<td>6M, 19B</td>
<td>16·7</td>
<td>0·41</td>
<td>16 (4n)</td>
</tr>
<tr>
<td></td>
<td>2n, 2n/4n, 4n†</td>
<td>19</td>
<td>10M, 9B</td>
<td>23·9</td>
<td>0·58</td>
<td>9 (no mitotic plates or not analysable)</td>
</tr>
</tbody>
</table>

* Transferred to CM as 2-cell.
† Transferred to CM as 3- or 4-cell or as pseudofragmenting.

and selection of embryos after 2–4 h of culture in CB-free medium) guaranteed high reliability of selecting 4n embryos, but presumed tetraploid eggs occasionally developed into diploid blastocysts (Table 3). This is to be expected in view of the asynchrony of the second cleavage (Table 4).

A majority of eggs which following CB treatment remained or became 3-cell (presumed mosaics) also developed into regular blastocysts with a slightly

**Figures 1–6**

Fig. 1. A group of 2-cell embryos from the experiment described in Table 4, at the end of 6 h treatment with CB. × 150.

Fig. 2. The same eggs after 1-20 h in CB-free medium. Note that with the exception of three embryos which have remained 2-cell and have retained smooth surface, the others either have resumed cleavage (in one or both blastomeres) or have undergone pseudofragmentation. At this moment the eggs were fixed and the state of their nuclear apparatus is described in the last column of Table 4. × 150.

Figs. 3 and 4. Tetraploid egg with both blastomeres binucleate (Fig. 3) and 2n/4n mosaic 3-cell egg (Fig. 4). These two eggs are from the group shown in Fig. 2. Permanent haematoxylin preparation. × 550.

Fig. 5. Mosaic blastocyst after 48 h in culture. × 400.

Fig. 6. Four tetraploid blastocysts with a distinct inner cell mass and two forming blastocysts. 52 h in culture. × 300.
Table 3. Karyology of tetraploid, mosaic and diploid embryos produced with CB from 2-, 3- and 4-cell eggs

<table>
<thead>
<tr>
<th>Original stage</th>
<th>Stage and presumed ploidy ( ) determined 2-4 h after CB treatment</th>
<th>Total no. of embryos</th>
<th>Number of morulae and blastocysts</th>
<th>No. of cells or mean no. of cells (x)</th>
<th>2n</th>
<th>2n &amp; 4n</th>
<th>4n</th>
<th>not analysable or no mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2 (4n)</td>
<td>46</td>
<td>9M, 37B</td>
<td>19-3 (x)</td>
<td>1</td>
<td>0</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>3 (2n/4n)</td>
<td>10</td>
<td>4M, 6B</td>
<td>22-1 (x)</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4 (2n)</td>
<td>2</td>
<td>2</td>
<td>2B</td>
<td>40, 44</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (2n/4n)</td>
<td>2</td>
<td>2</td>
<td>2B</td>
<td>26, 28</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4 (2n)</td>
<td>3 (2n/4n)</td>
<td>4</td>
<td>1M, 3B</td>
<td>24, 27, 28, 33</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4 (2n/4n)</td>
<td>3 (2n/4n)</td>
<td>4</td>
<td>4B</td>
<td>20, 47, 48, 58*</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Embryos cultured for 72 h.

Table 4. Rate of cleavage of control eggs and the state of nuclear apparatus of sister CB-treated 2-cell eggs during 7 h culture in vitro (experimental eggs examined in permanent preparations)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>3:30</th>
<th>5</th>
<th>6</th>
<th>7:20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM 6 x 2-cell</td>
<td>3 x 2-cell</td>
<td>1 x 2-cell</td>
<td>1 x 3-cell</td>
<td>1 x 3-cell</td>
<td>1 x 3-cell</td>
</tr>
<tr>
<td></td>
<td>2 x 3-cell</td>
<td>1 x 3-cell</td>
<td>1 x 3-cell</td>
<td>1 x 3-cell</td>
<td>1 x 3-cell</td>
</tr>
<tr>
<td></td>
<td>1 x 4-cell</td>
<td>4 x 4-cell</td>
<td>5 x 4-cell</td>
<td>5 x 4-cell</td>
<td>5 x 4-cell</td>
</tr>
<tr>
<td>CB 20 x 2-cell</td>
<td>1 x two mononucleate cells</td>
<td>1 x one mononucleate cell + one binucleate cell</td>
<td>4 x two binucleate cells</td>
<td>1 x two mononucleate cells</td>
<td>1 x telophase + binucleate cell</td>
</tr>
<tr>
<td></td>
<td>1 x two mononucleate cells</td>
<td>1 x two mononucleate cells</td>
<td>1 x four mononucleate cells*</td>
<td>1 x two mononucleate cells</td>
<td>1 x two mononucleate cells</td>
</tr>
<tr>
<td></td>
<td>15 transferred to CM</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2 x pseudofragmentation with 4 nuclei</td>
<td>2 x pseudofragmentation with 4 nuclei</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cleavage resumed in one or both blastomeres.
Tetraploid and 2n/4n mosaic mouse embryos

Fig. 7. Tetraploid metaphase plate from a cleaving egg. The sex chromosome constitution is \( XXYY \) (arrows point to \( Y \) chromosomes). \( \times 1400 \).

Fig. 8. Air-dried preparation of a mosaic blastocyst showing a diploid and a tetraploid mitosis. \( \times 200 \).

Fig. 9. Tetraploid metaphase plate from the egg-cylinder recovered on the 10th day.

higher number of cells than their tetraploid litter-mates (Fig. 5, Table 3). Mosaicism was karyologically confirmed in three embryos selected as presumed mosaics and also in three others which were left in culture as 3-4-cell or pseudo-fragmenting (Tables 2 and 3, Fig. 8). Karyological studies greatly underestimated the incidence of mosaicism, because the embryos contained too few cells (20–30) always to have both a 4n and a 2n metaphase plate.
Table 5. Postimplantation development of 2n (control), 4n and 2n/4n eggs transferred to pseudopregnant recipients

<table>
<thead>
<tr>
<th>Presumed ploidy</th>
<th>Site of transfer</th>
<th>No. of recipients</th>
<th>No. and stage of eggs</th>
<th>No. of recipients with implants</th>
<th>Implantations</th>
<th>Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total (%)</td>
<td>Embryos (%)</td>
</tr>
<tr>
<td>2n</td>
<td>oviduct</td>
<td>5</td>
<td>29 × 4-cell*</td>
<td>5</td>
<td>18 (62.0)</td>
<td>17 (58.6)</td>
</tr>
<tr>
<td></td>
<td>uterus</td>
<td>2</td>
<td>9 blastocysts†</td>
<td>1</td>
<td>4 (2)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>7</td>
<td>38</td>
<td>6</td>
<td>22§ (57.8)</td>
<td>19 (50.0)</td>
</tr>
<tr>
<td>4n</td>
<td>oviduct</td>
<td>9</td>
<td>43 × 2-cell</td>
<td>8</td>
<td>31 (72.1)</td>
<td>20 (46.5)</td>
</tr>
<tr>
<td>2n/4n</td>
<td>oviduct</td>
<td>3</td>
<td>16 × 3-cell‡</td>
<td>3</td>
<td>13 (81.2)</td>
<td>12 (75.0)</td>
</tr>
</tbody>
</table>

* Includes five eggs which stayed in DMSO for 4 h and cleaved from 2- to 4-cell, fifteen 2-cell eggs which resumed cleavage after CB treatment and nine eggs which were subjected to CB after they had divided into 4-cell.
† Blastocysts developed in vitro from 4-cell eggs treated with CB.
‡ Includes three 2-cell eggs which after CB treatment cleaved in CM into 3-cell; five 3-cell eggs which were 3-cell on placing them in CB and remained at this stage after treatment and eight 4-cell eggs which in CB reverted to the 3-cell stage.
§ Two embryos died on the 13-14th day.
|| Includes one small 10-day old triploid egg cylinder.

Table 6. Summary of postimplantation development of tetraploid embryos

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Main series of expts. – see Table 5</th>
<th>Embryos from additional expts.†</th>
<th>Total no. of available embryos</th>
<th>Way of examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Implantations</td>
<td>Embryos</td>
<td></td>
<td>Histology</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>3*</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>20</td>
<td>25</td>
<td>14</td>
</tr>
</tbody>
</table>

* One implantation contained numerous trophoblastic giant cells (cf. Fig. 14).
† Embryos developed from 4-cell tetraploid eggs. Number of implantations is not given because eggs were transplanted to pregnant animals.
Tetraploid and 2n/4n mosaic mouse embryos

Development after implantation

Results of transfers of experimental and control eggs are presented in Table 5. In all three groups of eggs implantation rate was above 50%. Postimplantation mortality was very low in 2n control and 2n/4n experimental embryos and high among 4n embryos – only 20 out of 31 implantations contained embryos. This group of 20 embryos was augmented by five additional egg-cylinders produced in a separate series (see Materials and Methods). The collected material is presented in Table 6.

Morphology of tetraploid embryos

6th day of pregnancy (5½-day embryos). Four normal embryos at an early egg-cylinder stage, with a very small cavity in the embryonic part (Fig. 10).

7th day. Four normal egg-cylinders with a proamniotic cavity and one small and retarded cylinder lacking a cavity and covered with high columnar endoderm. All embryos had well-developed ectoplacental cones. Numerous mitoses were present in all embryos, both in embryonic and extra-embryonic parts.

8th day. One very small egg-cylinder retarded by about 2 days. It lacked the proamniotic cavity and was covered by high columnar endoderm which in a few places formed ingrowing folds (Fig. 11). The two other egg-cylinders were retarded by one day and had abnormally small (short and narrow) embryonic part (Fig. 12). In both cylinders the proamniotic cavity did not extend to the upper end of the extra-embryonic part. The ectoplacental cones were well developed.

9th day. One egg-cylinder retarded by 1½ day (Fig. 13), with the embryonic part underdeveloped with a vestigial cavity. A small amnion was in the process of formation. The proamniotic cavity occupied only the lower half of the extra-embryonic part. The ectoplacental cone was well developed and the embryo was surrounded by numerous trophoblastic giant cells.

Six egg-cylinders varying in size and retarded in development by 1–2 days were examined only macroscopically. Embryonic structures were absent even in the largest cylinders. Three of them were studied karyologically and proved to be tetraploid.

10th day. Large egg-cylinder with foetal membranes of normal structure and typical for the 9th day (Figs. 15 and 16) and with a vestigial hind region of the embryo containing a small piece of unilaterally formed neural plate (Fig. 17). Embryonic mesoderm was very scanty. Yolk sac contained numerous blood islands and chorion has fused with the ectoplacental cone.

Among four dissected embryos there were three egg-cylinders of similar structure (two were studied karyologically and proved to be tetraploid, Fig. 9) and one abnormal and partial embryo consisting of the hind region and a tail. The approximate size of this egg-cylinder was about one-third of a control implant. Tetraploidy was confirmed karyologically. This case represents the most successful tetraploid development that we have so far observed.
Table 7. Karyology of 2n/4n implanted embryos

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Embryo no.</th>
<th>Tetraploid and hypotetraploid plates (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Embryo</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.4</td>
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<td>3.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of examined metaphase plates varied between 30 and 210.

11th day. One implantation filled with plentiful trophoblastic cells (small actively dividing and giant) and containing an abortive egg-cylinder (Fig. 14). One dissected egg-cylinder with a vestigial embryonic part attached to the bottom of the yolk sac. Tetraploidy confirmed karyologically.

2n/4n mosaic embryos

Eleven out of 12 embryos were morphologically normal and developing at a normal rate. One embryo recovered on the 10th day was retarded at the

Figures 10-17

Fig. 10. 5½-day (6th day of pregnancy) egg-cylinder of normal structure and size. × 300.

Fig. 11. 7½-day (8th day) retarded egg-cylinder with a small embryonic part and surrounded by high and folded endoderm. × 200.

Fig. 12. 7½-day egg-cylinder with retarded and disproportionally small embryonic part. × 100.

Fig. 13. 8½-day (9th day) retarded egg-cylinder with an underdeveloped embryonic part. Amnion has started to form. Proamniotic lumen is short and does not penetrate into the upper half of the extra-embryonic part.

Fig. 14. Group of trophoblastic giant cells filling the implantation cavity (11th day of pregnancy). Close to the dead egg-cylinder there is a group of actively dividing small cells most probably originating from the disorganized ectoplacental cone. × 100.

Figs. 15 and 16. Two sections of a 9½-day (10th day) egg-cylinder with normally formed foetal membranes and rudimentary hind region of the embryo. Fig. 15 is a section at the level of allantois, Fig. 16 is a section from a foremost region and shows well developed amnion and absence of the embryo. × 50.

Fig. 17. Transverse section through vestigial embryonic structures lying close to the base of allantois. Note unilaterally formed neural plate and scarcity of embryonic mesoderm; the same embryo as in Figs. 15 and 16. × 150.
stage of an early egg-cylinder and on karyological examination turned out to be triploid. From eight 10- and 13-day conceptuses chromosomal preparations were made separately from embryos and foetal membranes (Table 7). Tetraploid and hypotetraploid metaphase plates were detected in all conceptuses but only in five cases were they found in embryos and in a very low proportion (below 4 %). The incidence of tetraploid mitoses in foetal membranes was in four cases also very low (0·5–3·6 %) but in four others was very high and fluctuated between 25 and 50 %.

Control diploid embryos

Embryos were dissected on the 10th, 17th and 18th day and subjected to external examination only. Two embryos died around 13–14th day. All others looked perfectly normal.

DISCUSSION

CB as a tool of suppressing cytokinesis of the 2nd cleavage

The paper of Snow (1973) and the present study show that CB at a concentration of 10 μg/ml is highly effective in producing tetraploidy by suppression of the 2nd cleavage. Our experimental system produces 50 % of tetraploid embryos, together with 20 % of 2n/4n mosaic embryos. The mosaic embryos can be produced from 2-, 3- and 4-cell eggs but the way of their origin as well as their incidence is different in each of these stages (cf. Table 1 and p. 51).

The main factor which in our experiments limited the effectiveness of CB in producing tetraploid embryos was the asynchrony of the 2nd cleavage – both between eggs (intra-batch variation) and between blastomeres. This double asynchrony is well illustrated by the results of one experiment described in Table 4. Our observations do not confirm the opinion of Perry and Snow (1975) that ‘development within litters is closely synchronous at the 2- to 4-cell stage’.

The experimental procedure worked out by Snow and by us, although identical in principle, differed in the duration of treatment with CB which was much shorter in our experiments (3–8½ h (average time 4½ h) versus 12 h). This is probably the reason that we have not observed the harmful effects of CB, such as suppression of consecutive cytokineses leading to high degrees of ploidy, which occurred in 10–15 % of eggs in Snow’s experiments (Snow, 1973).

The fact that application of CB at the time of cleavage or shortly afterwards causes regression of the furrow, shows that the presence of the drug before the initiation of cytokinesis is not required for its suppression. This is in agreement with the findings for Arbacia (Schroeder, 1972) and Xenopus (Bluemink, 1971). Thus the ‘effective time’ of treatment is only the period from the scheduled moment of initiation of cytokinesis onwards. However, if the arrested cleavage is not to be resumed, treatment must continue for a certain period of time (half hour appears to be a minimum, Opas, 1977).

It is generally accepted that the short-lasting contractile ring promotes cyto-
kinesis in animal cells (Rappaport, 1975; Schroeder, 1975). This structure, first described by Schroeder (1968), is CB-sensitive and under influence of the drug either disappears (Arbacia eggs, Schroeder, 1972) or undergoes disarrangement (Xenopus eggs, Luchtel et al. 1976). The contractile ring in cleaving mammalian eggs has received very little attention up to now (Szollosi, 1968, 1970; Gulyas, 1973) and the effect of CB on this structure at the ultrastructural level has not been investigated at all. We believe that our observations yield some information on the temporal organization of this contractile system in mouse blastomeres. The fact that the blastomeres removed from CB can resume cleavage long after karyokinesis had been completed, suggests that either the division apparatus is not completely degraded and can be ‘repaired’ or, the blastomeres retain for a certain period of time the ability to construct it de novo. Variable behaviour of blastomeres following CB treatment could be accounted for as follows: if a cell is subjected to CB until after this ability to construct contractile apparatus is lost then it retains regular shape and smooth surface; if, however, treatment is completed earlier, then resumption of cleavage or pseudofragmentation occur. We believe that the violent surface activity – irrespective of whether it results in resumption of cleavage or in transitory pseudofragmentation – represents ‘attempts’ of the blastomere to complete cytokinesis due to retention or re-formation of the contractile system.

**Developmental effects of tetraploidy**

The main effect of the suppression of the 2nd cleavage on preimplantation development of tetraploid embryos was the reduction of the number of cells at the blastocyst stage to about 40% of that in control diploids. Identical observations were made by Snow (1975). According to Snow (1975, 1976), most tetraploid embryos develop into blastocysts with very few inner mass cells or even into trophoblastic vesicles. According to our observations all embryos that have undergone cavitation were true blastocysts (with ICM); no efforts were made, however, to estimate the number of ICM cells. Absence or small size of ICM is considered by Snow to be the main cause of the heavy mortality of tetraploid embryos which he has observed shortly after implantation. While this interpretation appears to be correct in the case of his material, it does not apply to our material in which early resorptions were rare. The survival of 4n embryos during the first three days of postimplantation pregnancy was in these two studies strikingly different: 6 embryos in 31 implantations (Snow, 1976) as compared to 9 in 10 (this study). This difference may be due to the fact that Snow transferred to recipients blastocysts which had developed from 2-cell eggs in vitro, while we transplanted 2-cell eggs which had been cultured for up to 12 h only and which continued the rest of preimplantation development in vivo. Although the structure and the cell number of these blastocysts were not examined, it can be safely postulated that under physiological conditions they have attained by the time of implantation higher number
of cells than those developing in vitro. Although in principle we agree with Snow that tetraploid blastocysts must have a reduced ICM (reduction of the total number of cells always affects in the first instance the size of ICM, Tarkowski and Wróblewska, 1967), we believe that if optimal conditions are provided, the tetraploid blastocysts need not have any developmental problems at the time of implantation.

Although shortly after implantation the viability of our $F_1$ (CBA × C57BL) × A tetraploid embryos was high and most were morphologically normal though some slightly retarded, their development was clearly affected from the 8th day of pregnancy. The first symptom of the developmental defect is the retardation of the development of the embryonic part of the egg-cylinder. From this moment the growth of the whole conceptus slows down, but formation of foetal membranes may be initiated and in some conceptuses is successfully accomplished. The yolk sac and the ectoplacental cone appear to be least affected. Formation of amnion and allantois implies that mesoderm is being produced. The embryonic part is either completely arrested and forms a clump of tissue suspended to the yolk sac or, at best, gives rise to rudimentary hind region of the embryo. The main developmental problem appears to be inadequate production of mesoderm, which is generally very scanty and utilized mainly by the foetal membranes. The competence of ectoderm to neural induction is not impaired, as shown by the presence of neural structures in the two most advanced partial embryos.

Although the examined egg-cylinders varied both in size and structure the developmental anomaly which they displayed was basically consistent. This anomaly is not unique for tetraploid embryos of this genetic constitution because it was also described in CBA triploid embryos (Wróblewska, 1971). Also all human tetraploid conceptuses described so far consisted of foetal membranes only (Carr, 1971; Hamerton, 1971).

Contrary to our findings, Snow (1975) has provided ample evidence that in the randomly bred Q strain about 20% of implanted tetraploid blastocysts develop advanced embryos which can survive even until birth. The case of the recently recorded tetraploid infant (Golbus et al. 1976) shows that in Man also this aberration is occasionally compatible with embryogenesis and even development beyond birth. This different developmental potential of mouse and human tetraploid embryos is puzzling and corroborates the conclusion inferred from studies on triploidy (cf. Wróblewska, 1971), that morphological expression of polyploidy in mammals is very variable and is probably modified by the genetic background on which it occurs.

The opinion of Snow (1975) that developmental problems encountered by tetraploid embryos are physiological and numerological in nature rather than genetic may be correct as an explanation of slight morphological and histological defects in advanced embryos, but does not seem to be an answer to the failure of embryogenesis of the type that we have observed. Whether the
deficiency of embryonic mesoderm which appears to be the direct cause of faulty embryogenesis or of its absence, is simply due to the reduced rate of proliferation of tetraploid cells, or has a more complex background, cannot be at present answered. That tetraploid cells are generally less viable than diploid cells is shown by the fact that in 2n/4n mosaics examined on the 10th and 13th day of pregnancy tetraploid cells were practically eliminated from the embryos, though not necessarily from the foetal membranes. The absence of tetraploid component in mice developed from mosaic embryos (Graham, 1971) also supports this conclusion. This may account for the exceptionally rare incidence of diploid/tetraploid mosaicism in Man (Kohn, Mayall, Miller & Mellman, 1967; Kelly & Rary, 1974).

We thank Mrs Joanna Wróblewska for making chromosomal preparations from implanted embryos and Dr W. Ożdżeński for help in analysing histological preparations of tetraploid embryos.

REFERENCES


(Received 5 January 1977, revised 24 April 1977)