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Parthenogenesis and cytoskeletal organization in ageing mouse eggs

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SUMMARY

The cytoskeletal organization of the mouse egg changes during ageing *in vivo* and *in vitro*. The earliest change observed is the disappearance of the microfilament-rich area overlying the meiotic spindle. This is followed by the migration of the spindle towards the centre of the egg. Finally the spindle breaks down and the chromosomes are no longer organized on a metaphase plate. This spindle disruption may result from changes in the microtubule nucleating material found at the spindle poles and from an increase in the critical concentration for tubulin polymerization. It is possible to correlate the changes in the cytoskeletal organization of the egg occurring during ageing with the different types of parthenogenetic embryos obtained after ethanol activation. These observations strengthen the hypothesis that the actin-rich cortical area that overlies the meiotic spindle forms a domain to which the meiotic cleavage furrow is restricted and provides some insights into the mechanisms by which different types of parthenogenetic embryos are generated.

INTRODUCTION

The first few hours after fertilization of the mouse egg are characterized by major changes in cytoskeletal organization (for review see Maro, 1985) involving both microfilaments (Maro, Johnson, Pickering & Flach, 1984; Longo & Chen, 1985; Maro, Johnson, Webb & Flach, 1986; Van Blerkom & Bell, 1986) and microtubules (Maro, Howlett & Webb, 1985*b*; Schatten, Simerly & Schatten, 1985) that are essential for the formation of the biparental diploid genome of the embryo. The resumption of meiosis results in an unequal cleavage leading to the formation of two cells, very dissimilar in size: the polar body being about 100-times smaller than the egg. This unequal cleavage allows the egg to retain most of the constituents synthesized during oogenesis. We reported previously on the close association between the cytocortex and both the meiotic female and newly introduced male chromosomes, and demonstrated that in both cases the overlying

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membrane was associated with a subcortical focus of stable polymerized actin (Maro *et al.* 1984; see also Fig. 1A,B). Moreover, when the female chromosomes were dispersed with nocodazole (Maro, 1985; Maro *et al.* 1986) or additional chromosomes were injected into the egg (Van Blerkom & Bell, 1986), each of the dispersed chromosome clusters was associated with an actin-rich cortical domain, leading to the suggestion that the chromosomes were able to induce these cortical changes. We suggested also that the meiotic spindle was maintained in a peripheral position by the microfilament network and that the development of the cleavage furrow was limited to the actin-rich domain of the cortex, thereby yielding unequal cleavage (Maro *et al.* 1984, 1986). In order to test this hypothesis, we have investigated how cytoskeletal organization changes with the ageing of the egg and what relationship these changes bear to the ease and type of parthenogenetic activation. It is known that the type of parthenogenetic embryo obtained by activation does change in relation to the postovulatory age of the egg (Kaufman, 1973). Thus, when eggs are activated at 16–20 h post-hCG most of the parthenogenetic embryos have a second polar body and one pronucleus, whilst activation at 25 h post-hCG generally yields two cells of similar size (termed immediate cleavage). If cleavage is indeed dependent upon the organization of the cytocortical actin, we might expect the latter to be modified in ageing eggs and so to explain the different types of parthenogenetic embryos obtained.

MATERIALS AND METHODS

Recovery of eggs

Eggs were recovered from 3- to 5-week-old (C57B1.10×CBA) F_1 or MF1 mice after superovulation with 5 i.u. pregnant mares' serum (PMS, Intervet) followed 48 h later by 5 i.u. human chorionic gonadotrophin (hCG, Intervet). The females were killed either at 12, 16, 20, 24, 28 or 32 h post-hCG and the ovulated eggs released from the oviducts into pre-equilibrated drops of Whittingham's medium (Whittingham, 1971) containing 30 mg BSA ml^{-1}.

Parthenogenetic activation

Ethanol was used to artificially activate the eggs (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982). Parthenogenetic activation of the eggs was performed as described in Kaufman (1982) except that PBS was replaced by medium 2 containing 4 mg ml^{-1} bovine serum albumin (M2+BSA; Fulton & Whittingham, 1978). Eggs were activated by a 4·5 min exposure of the cells to a 7% ethanol solution in M2+BSA then washed three times in M2+BSA and cultured in Whittingham's medium (Whittingham, 1971).

In order to score as accurately as possible the types of parthenogenetic embryos obtained after ethanol activation, the zonae pellucidae were kept intact in order to be able to assess unequivocally whether a second polar body was present in those embryos in which only one pronucleus was present. The presence of the zona pellucida reduces adhesivity, therefore after being freed of their cumulus cells, eggs and embryos were placed in chambers that had been coated first with a 1 mg ml^{-1} poly-L-lysine (PLL; Sigma) solution, dried and finally coated with a 0·1 mg ml^{-1} Concanavalin A (Con A; Miles) solution in phosphate-buffered saline (PBS). This treatment ensures that eggs within their zona pellucida stick to the glass chambers. Eggs and embryos were then fixed and stained with Hoechst dye before examination under the fluorescence microscope.

Cell fixation and immunocytological staining

When necessary, eggs were removed from their cumulus cells by brief exposure to 0·1 M-hyaluronidase (Sigma), and were freed from their zonae pellucidae by brief exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975), followed by a rinse in M2+BSA. After removal of the zonae pellucidae, eggs were then placed in specially designed chambers as described previously (Maro *et al.* 1984).

The cells were then fixed in one of two ways:

(i) For actin staining with affinity-purified rabbit anti-actin polyclonal antibodies (Gounon & Karsenti, 1981) cells were fixed for 30–45 min with 3·7 % formaldehyde (BDH) in phosphate-buffered saline (PBS), washed in PBS then extracted for 10 min in 0·25 % Triton X-100 (Sigma) and washed in PBS.

(ii) For MTOC staining with a human anti-pericentriolar material (PCM) serum (Calarco-Gillam *et al.* 1983) cells were extracted for 10 min in HPEM buffer (10 mM-EGTA, 2 mM-$MgCl_2$, 60 mM-Pipes, 25 mM-Hepes, pH 6·9; Schliwa, Euteneuer, Bulinsky & Izant, 1981) containing 0·25 % Triton X-100, washed in HPEM buffer and fixed for 30 min at 37°C with 3·7 % formaldehyde in HPEM buffer.

Immunocytological staining was performed as described in Maro *et al.* (1984). In order to stain the chromosomes, fixed cells were incubated in Hoechst dye 33258 (10 $\mu g\,ml^{-1}$ in PBS) for 20 min at 20°C.

Photomicroscopy

The coverslips were removed from the chambers and samples were mounted in 'Citifluor' (City University, London) to reduce fading of the reagents and viewed on a Leitz Ortholux II microscope with filter sets N2 for rhodamine-labelled reagents, L2 for fluorescein-labelled reagents and A for Hoechst dye. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orthomat photographic system.

RESULTS

Ethanol activation of aged eggs

For this study we used eggs that were aged either *in vivo* or *in vitro*. In both cases it was possible to activate parthenogenetically the eggs, but the maximum percentages of activated embryos was higher in eggs aged *in vivo* than in eggs aged *in vitro* (Table 1). For this reason the changes occurring in the groups of eggs aged *in vivo* will be described although similar qualitative results were obtained after ageing *in vitro* (see below).

Different types of parthenogenetic embryos were observed during this study (Fig. 1) and, as previously described by Kaufman (1973), there was a high incidence of embryos with one polar body and one pronucleus when young (12–20 h post-hCG) eggs were activated (Fig. 1A), while immediate cleavage (Fig. 1C) was observed in most cases when older (24–28 h post-hCG) eggs were treated with ethanol (Fig. 2, upper panel). During the 16–32 h post-hCG period, a small group of embryos with two pronuclei was observed (Fig. 1B). In old eggs (32–36 h post-hCG), ethanol treatment mostly induced the formation of embryos with one pronucleus and no polar body (Fig. 1D). During the same period a greater proportion of embryos were fragmented, activation leading to cell death (Fig. 2, upper panel).

Very similar results were observed when eggs aged *in vitro* were activated by ethanol (Fig. 3, upper panel), the main difference observed between the two groups of eggs being a slower time course of the shift in the type of parthenogenote generated. Also, we did not observe fragmented embryos, even after eggs had been aged *in vitro* to the equivalent of 48 h post-hCG.

Changes in the cytoskeletal organization of aged eggs

The localization of actin in eggs aged *in vivo* or *in vitro* was similar. In young eggs, an actin-rich area overlying the meiotic spindle was present (Fig. 4A,B). Later, the cortical actin became evenly distributed around the cell cortex with the spindle still in a peripheral position (Fig. 4C,D). Later still, the spindle migrated towards the centre of the egg (Fig. 4E,F). A small group (<10 %) of eggs with an actin-deficient area overlying a peripheral spindle was observed in very old eggs (Fig. 4G,H). This group probably corresponds to the dying eggs. These results are summarized in Fig. 5.

We studied also the organization of the spindle by staining the chromosomes with the Hoechst dye and by immunolabelling of the spindle poles with a serum that recognizes the pericentriolar material (PCM; Calarco-Gillam *et al.* 1983; Maro

Table 1. *Activation rates of the mouse eggs used in this study*

Egg age (h post-hGC)	Ethanol activation: Egg number	Activated eggs: Number	Activated eggs: %	Number of eggs examined for: Actin and spindle localization	Number of eggs examined for: Spindle organization
Ageing *in vivo*					
12 h	260	59	22·7	111	59
16 h	253	183	72·3	93	70
20 h	198	183	92·4	136	71
24 h	169	155	91·7	105	53
28 h	160	144	90·0	121	85
32 h	89	79	88·8	72	45
36 h	66	41	62·1	55	27
Ageing *in vitro**					
24 h	200	123	61·5	175	97
36 h	96	70	72·9	138	101
48 h	103	44	42·7	141	119
Total	1594			1147	727

* For ageing *in vitro*, the eggs were recovered from the mice at 12 h post-hCG. Thus, the 12 h group of eggs aged *in vitro* is identical to the 12 h group of eggs aged *in vivo*. Moreover, the two types of ageing experiments (*in vivo* and *in vitro*) were performed on groups of mice of identical age that were injected with hormones simultaneously and cultured in the same batches of media. The numbers of eggs correspond to the total from two or three independent experiments. The data from each of the experiments were pooled, as similar results were observed (in Figs 2, 3 and 5).

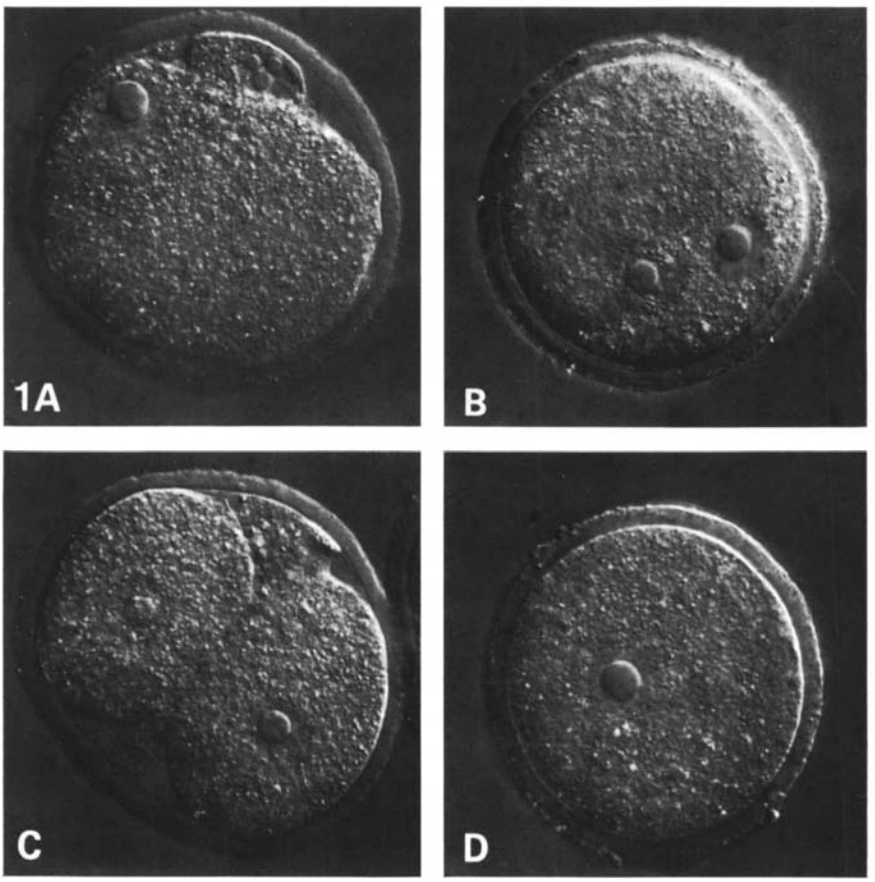


Fig. 1. Types of parthenogenetic embryos obtained after ethanol activation: one pronucleus and a second polar body (A), two pronuclei (B), immediate cleavage (C) and one pronucleus without second polar body (D). ×600.

et al. 1985*b*). The spindle poles of the mouse egg do not possess any centrioles, but are formed by two broad bands of PCM (Szollosi, Calarco & Donahue, 1972; Calarco-Gillam *et al.* 1983) constituted by several foci of PCM (Maro *et al.* 1985*b*). During ageing, we observed a disruption of the spindle (Fig. 6E–H), the poles being disorganized first (Fig. 6A–D). These changes in spindle organization occurred mainly in eggs in which the chromosomes were located centrally.

This disruption of the spindle and its poles during ageing is accompanied by changes in the polymerization threshold for tubulin and in the number of microtubule-organizing centres (MTOCs). Several cytoplasmic MTOCs are present in the subcortical area of the egg, in addition to the ones located at the spindle poles (see Fig. 6; Maro *et al.* 1985*b*). These cytoplasmic MTOCs do not normally nucleate microtubules in the metaphase II arrested egg because the critical concentration for tubulin polymerization is too high in the cytoplasm. Only in the vicinity of the chromosomes is the critical concentration for tubulin polymerization

low enough to allow microtubule nucleation, thereby allowing spindle formation (Maro *et al.* 1985*b*, 1986). However, it is possible to induce nucleation around the cytoplasmic MTOCs by use of taxol, a drug which decreases the critical concentration for tubulin polymerization (Schiff, Fant & Horwitz, 1979). During ageing, the number of cytoplasmic MTOCs decreased (Table 2). Moreover, this

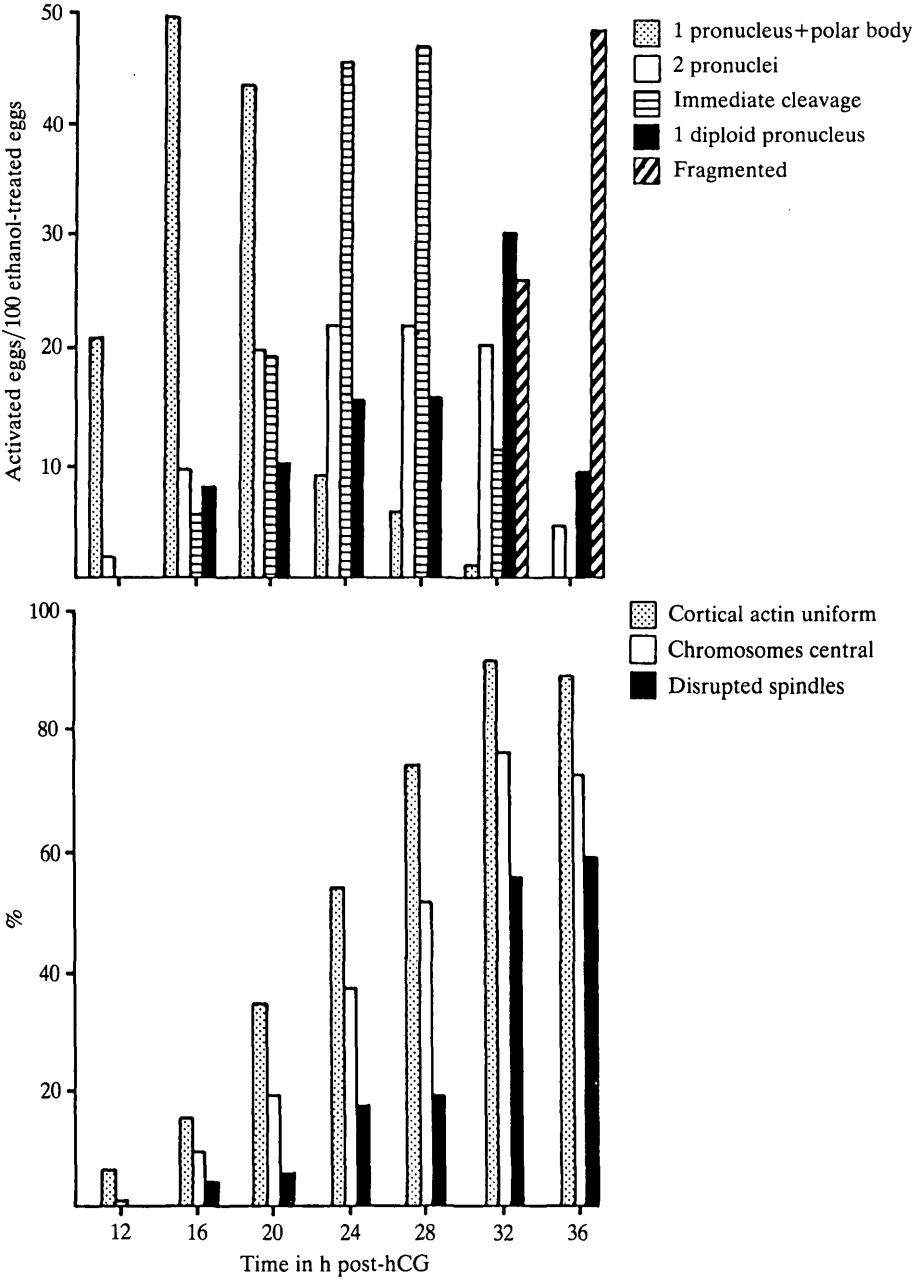


Fig. 2. Type of parthenogenetic embryos obtained after ethanol activation (upper panel) and cytoskeletal changes (lower panel) during ageing *in vivo*.

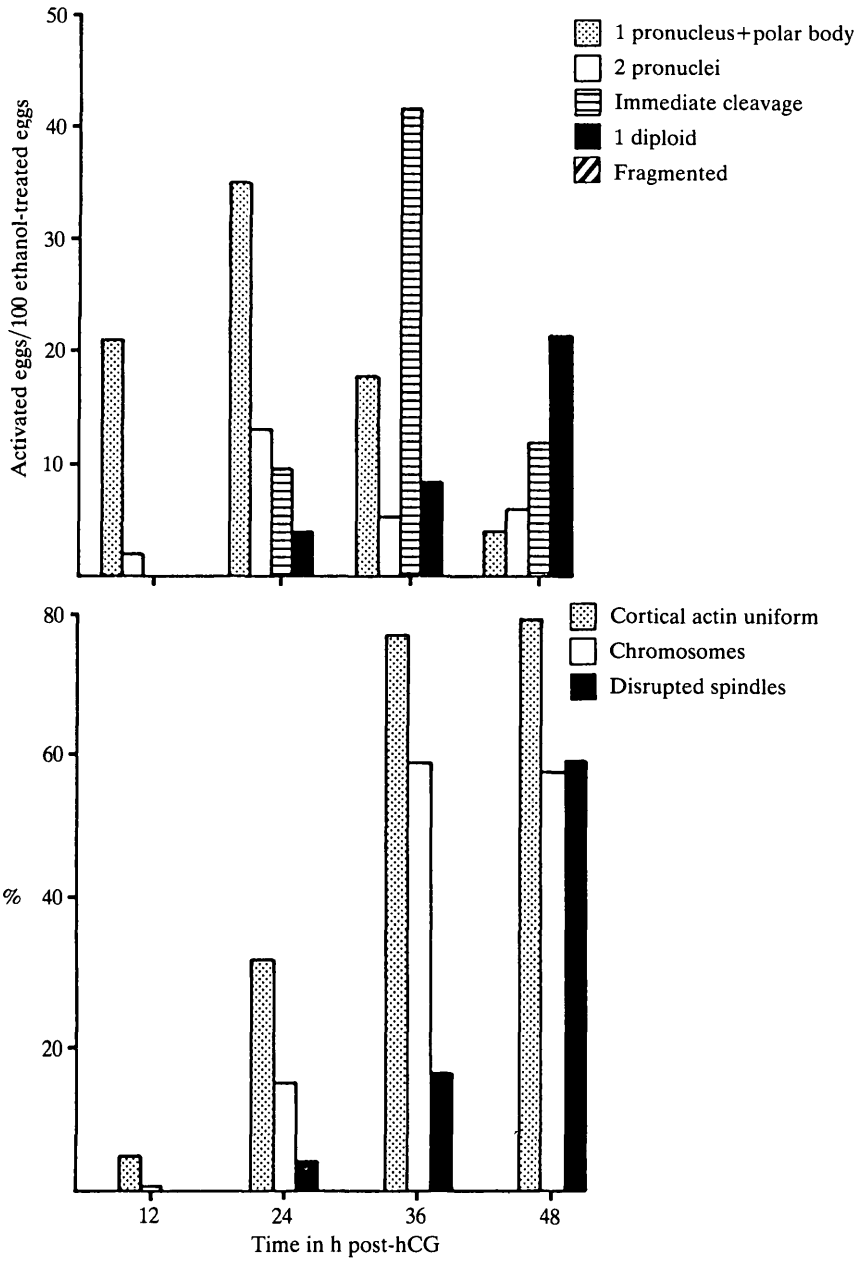
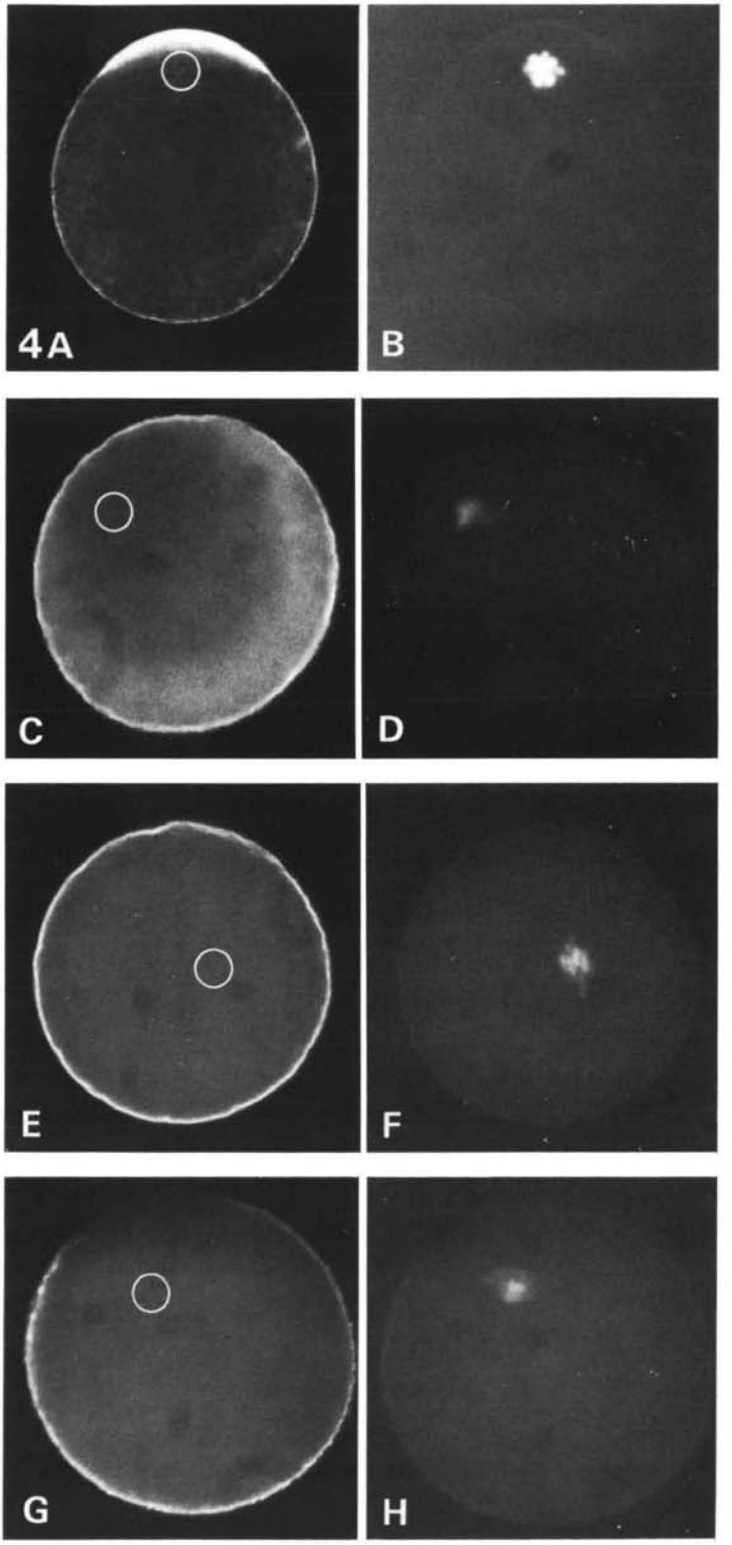


Fig. 3. Type of parthenogenetic embryos obtained after ethanol activation (upper panel) and cytoskeletal changes (lower panel) during ageing *in vitro*.

decrease in the number of observable MTOCs was associated with an increase in the cytoplasmic critical concentration for tubulin polymerization around the remaining MTOCs, as evidenced by the use of taxol. In older eggs, higher doses of taxol were necessary to induce the growth of microtubules around both the cytoplasmic MTOCs and the chromosomes (Table 3).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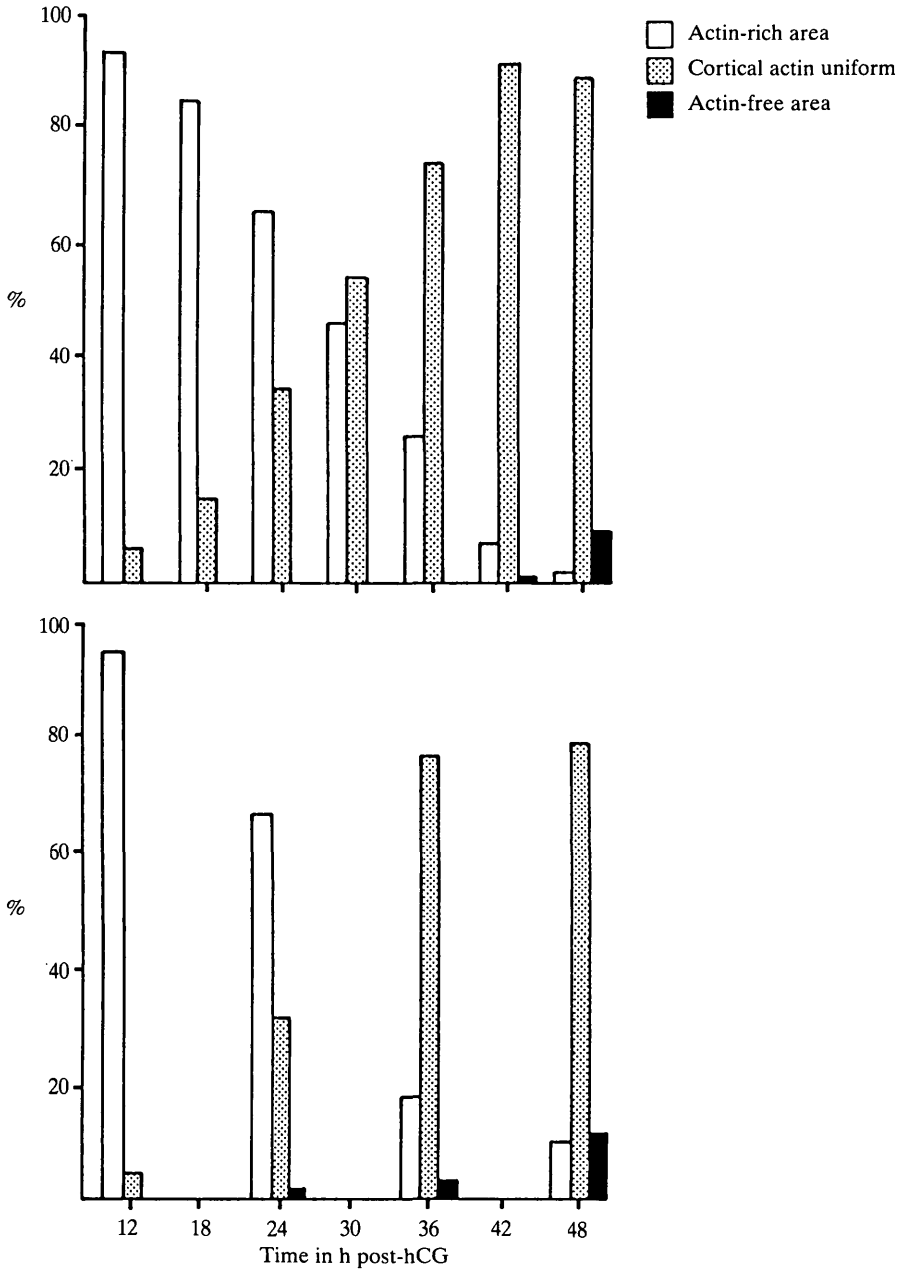

Fig. 5. Actin distribution during ageing *in vivo* (upper panel) and *in vitro* (lower panel).

Fig. 4. Eggs double stained for actin (A,C,E,G) and chromatin (B,D,F,H). Circles in A,C,E,G indicate the position of the chromosomes. (A,B) Peripheral chromosomes overlain by an actin-rich area. (C,D) Peripheral chromosomes with uniform distribution of cortical actin. (E,F) Central chromosomes with uniform cortical actin. (G,H) Actin-deficient area overlying the chromosomes. ×400.

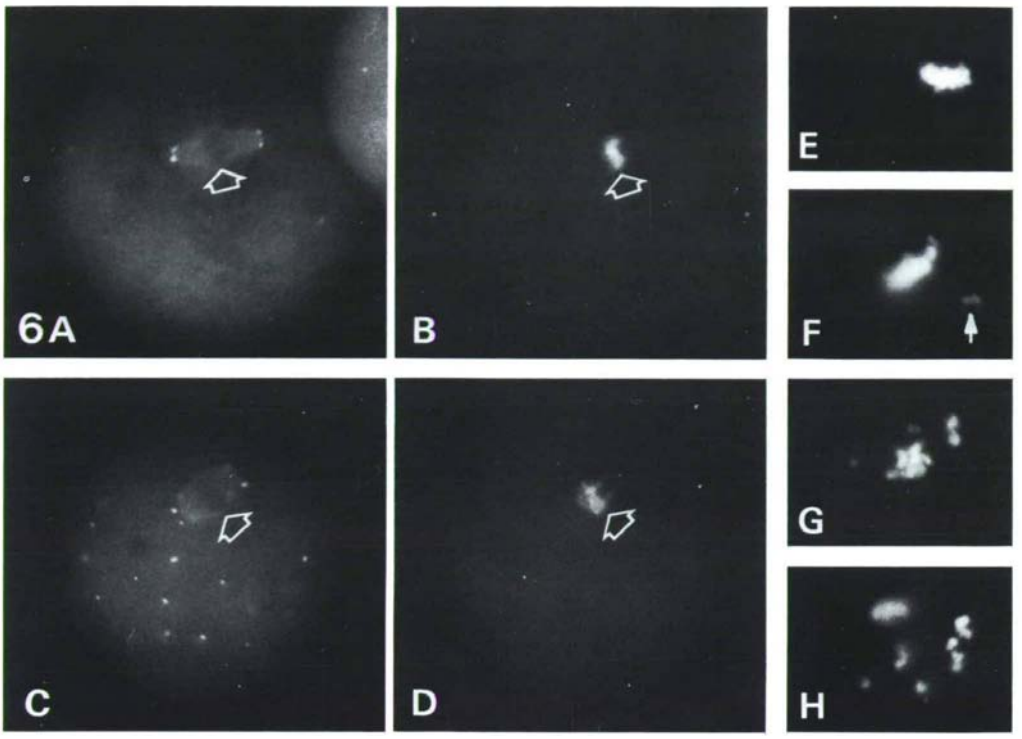

Fig. 6. (A–D) Eggs double stained for PCM (A,C) and chromatin (B,D), showing a normal spindle (A,B) and a loss of PCM at the spindle poles (C,D). Arrows indicate the position of the metaphase plate. ×400. (E–H) Spindle disruption as shown by staining of the chromatin with the Hoechst dye 33258. Note normal metaphase plate (E) and dispersed chromosomes (F–H). Arrow in F points to a pair of bivalent chromosomes detached from the metaphase plate. ×600.

The relationship between the type of parthenogenetic embryo obtained after ethanol activation and the cytoskeletal organization of the egg

In the case of the eggs aged *in vivo* it is possible to correlate the type of embryo obtained after activation with the cytoskeletal organization within the egg (Fig. 2, compare lower and upper panels).

(i) During the 12–20 h period following the hCG injection, most of the eggs possessed an actin-rich domain overlying a peripheral spindle. Over the same period the majority of the parthenogenetic embryos obtained had one pronucleus and a polar body.

(ii) During the 24–28 h period, many eggs had a central spindle and evenly distributed cortical actin. Immediate cleavage occurs most frequently during this period.

(iii) The percentage of parthenogenetic embryos with two pronuclei corresponded to the percentage of eggs with a peripheral spindle and evenly distributed cortical actin during the period 12–32 h.

(iv) The percentage of parthenogenetic embryos with only one pronucleus corresponded to the percentage of eggs with a disrupted spindle during the period

Table 2. *Fate of cytoplasmic MTOCs during ageing* in vitro

Egg age (h post-hCG)	Egg number	Mean number of MTOCs (±S.E.) in eggs with	
		Normal spindles	Disrupted spindles*
14 h	31	14·1 ± 4·3	—
20 h	44	11·3 ± 3·8	—
26 h	103	9·6 ± 3·8	10·2 ± 4·4
32 h	84	7·4 ± 3·9	10·9 ± 3·2
38 h	36	5·2 ± 2·8	9·2 ± 3·2
50 h	57	—	4·5 ± 2·1

*Note that the number of cytoplasmic MTOCs is larger in eggs with a disrupted spindle because of the dispersal of the MTOCs derived from the spindle poles. A similar increase may be achieved after experimental disruption of the spindle with nocodazole (Maro *et al.* 1985*b*).

Table 3. *Microtubule nucleation in eggs aged* in vitro

Egg age (h post-hCG)	Incidence of polymerized microtubules around either MTOCs* or chromosomes* in eggs treated with					
	1·5 μM-taxol			6·0 μM-taxol		
	Egg number	MTOCs	Chromosomes	Egg number	MTOCs	Chromosomes
14 h	54	96·3 %	100·0 %	64	100·0 %	100·0 %
20 h	56	100·0 %	100·0 %	53	100·0 %	100·0 %
26 h	69	85·5 %	92·8 %	48	85·4 %	75·0 %
32 h	70	77·1 %	94·3 %	37	70·3 %	94·6 %
38 h	27	37·0 %	44·4 %	21	76·2 %	95·2 %

*In the experimental conditions used, microtubules are only observed as large bundles around the chromosomes and, or, as asters around the MTOCs. The design of these conditions is described in detail in Maro *et al.* (1985*b*).

12–32 h. At 36 h this percentage was lower, but we must note that, at that time, most of the parthenogenetic embryos obtained were fragmented.

Similar observations were made with the groups of eggs aged *in vitro* (Fig. 3), although the changes observed were slower than in the corresponding eggs aged *in vivo*.

DISCUSSION

In this study we have demonstrated that the organization of the cytoskeleton changes during ageing of the mouse egg. Similar changes occur both during *in vivo* and *in vitro* ageing. Initially, the microfilament-rich area that overlies the meiotic spindle disappears, then the spindle migrates towards the egg centre. Finally the spindle breaks down and the chromosomes are no longer organized on a metaphase plate. These observations suggest that the movement of the spindle towards the centre of the egg may be secondary to a loss in the actin-rich area overlying the meiotic spindle and that the peripheral location of the spindle is maintained as long as this domain of the cell cortex rich in microfilaments is present (Maro *et al.* 1984, 1986; Longo & Chen, 1985; Van Blerkom & Bell, 1986). These conclusions are in

agreement with previous studies that showed that both the movement of the spindle towards the periphery of the meiosis I oocyte after germinal vesicle break down (Longo & Chen, 1985) and the dispersion of the chromosomes around the cell cortex after spindle disruption in mature eggs (Maro *et al.* 1986) are dependent upon microfilaments. It has been shown that the meiotic chromosomes are able to induce the changes in actin polymerization and organization that are responsible for the formation of the cortical domain rich in microfilaments and that this inducing activity appears approximately 60 min before germinal vesicle break down, as tested by the injection of chromosomes (Van Blerkom & Bell, 1986), and is lost after fertilization upon entry into interphase (Maro *et al.* 1984, 1986). Our results here show that this inductive capacity or the ability of the cytoskeletal elements to respond to it is lost during ageing in 50 % of the eggs by 24 h post-hCG and in all the eggs 12 h later. Most of the changes occurring in the pattern of protein synthesis after fertilization are related to the M-phase to interphase transition (Maro, Howlett & Johnson, 1985*a*; Howlett, 1986) and the polypeptides characteristic of M-phase continue to be synthesized during ageing. A single polypeptide (of 45×10^3 relative molecular mass), with an unusually short half-life, disappears shortly after ovulation, irrespective of fertilization, that is by about 20 h post-hCG in the ageing egg (Howlett & Bolton, 1985). It is therefore possible that this polypeptide might be involved in the maintenance of the microfilament-rich area of the cytocortex.

The only differences observed between *in vivo* and *in vitro* ageing are related to an earlier occurrence *in vivo* of the changes taking place during ageing and to the larger number of fragmented embryos after activation of old eggs aged *in vivo*. It is also clear that, when embryos are cultured *in vitro*, development is slowed down. These differences may be related to a decreased metabolic activity *in vitro* since our culture conditions are not able to recreate perfectly the uterine environment.

The movement of the spindle (at 14–18 h postovulation) and later its disorganization (at 18–24 h postovulation) have been observed qualitatively at the electron microscope level (Szollosi, 1971, 1975). It was suggested that the spindle disruption could be caused first by a 'less firm polar anchoring, possibly resulting from the absence of centrioles' (Szollosi, 1975). In our study we did observe early changes in the organization of the spindle poles, occurring before complete disruption of the spindle. In addition we observed a decrease in the total number of detectable MTOCs, corresponding to a loss of some of the PCM present in the egg. Moreover, those MTOCs remaining were less able to nucleate microtubules, because of an increase in the critical concentration for tubulin polymerization. These two properties would explain the subsequent disruption of the spindle. This physiological disruption of the spindle in ageing eggs (Szollosi, 1971, 1975; and our data) may also lead to aneuploidy and embryo loss if fertilization is delayed too long after ovulation.

It is possible to correlate the changes in the cytoskeletal organization observed with the different types of parthenogenetic embryos obtained after ethanol activation. Ethanol activation for 4·5 min by itself does not seem to favour one

particular type of parthenogenote, as is the case for certain other stimuli (for review see Kaufman, 1983), and it provides a very reliable and reproducible method of activation in contrast with some other stimuli that we have tested (our unpublished observations). Ethanol has been shown to induce aneuploidy as a result of non-disjunction that may involve any of the chromosomes (Kaufman, 1982, 1983) but does not seem to interfere with the formation of the polar body since, after ethanol activation, almost all of the young activated eggs form a polar body. It is only in aged eggs that polar body formation is impaired. The possible pathways to the formation of different types of parthenogenetic 1-cell embryos are schematized in Fig. 7. It is clear that after activation the normal formation of a

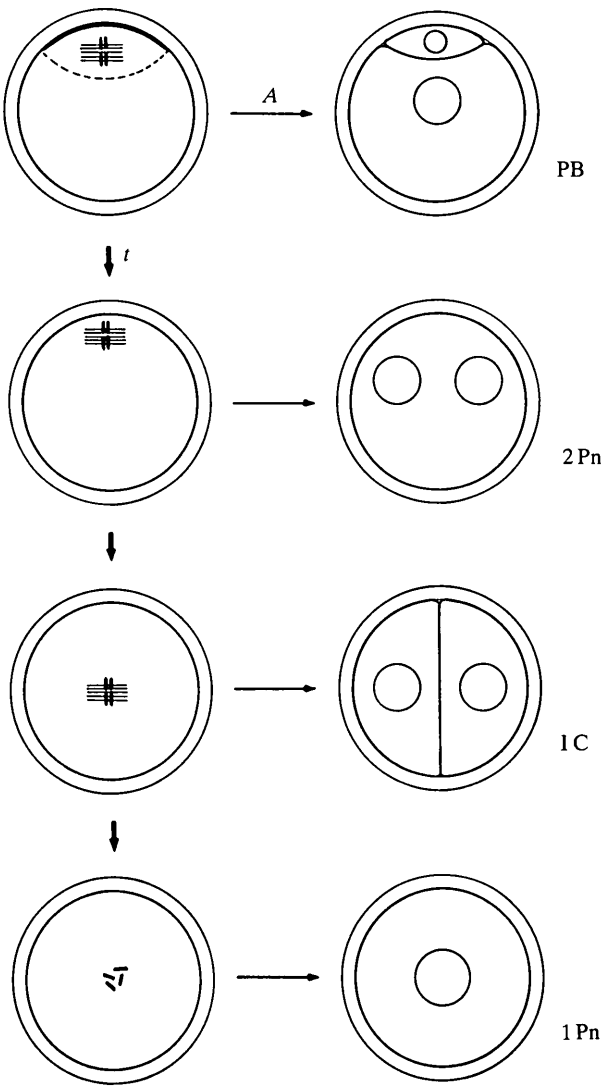

Fig. 7. Possible pathways of formation of parthenogenetically activated eggs. *A*, activation; *t*, ageing time.

polar body is related to, and therefore may be dependent upon, the existence of a microfilament-rich domain overlying the spindle. In the absence of this domain, with the actin distributed uniformly, a successful cleavage seems possible only if the spindle is intact and centrally located within the egg. Embryos with two pronuclei seem to derive from eggs in which the spindle had still been peripheral but the cortical actin had become uniform. Finally embryos with one single pronucleus may derive from eggs in which the central spindle was destroyed.

These observations strengthen the hypothesis that the actin-rich cortical area that overlies the meiotic spindle forms a domain to which the meiotic cleavage furrow is restricted and which maintains the spindle in a peripheral location, thus allowing polar body formation. They also help us to understand some of the mechanisms by which different types of parthenogenetic embryos are generated after ethanol activation. These observations may also give some clues as to how the formation of particular types of parthenogenotes might be favoured by some other parthenogenetic stimuli (see Kaufman, 1983).

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