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The origin of the nascent blastocoele in preimplantation mouse embryos: ultrastructural cytochemistry and effect of chloroquine

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Summary. Mouse morulae are known to undergo cavitation as soon as some external cells have entered the sixth cell cycle (Garbutt et al. 1987). Since the early cytological features of cavitation are still unclear, we undertook a careful ultrastructural analysis of late morulae-nascent blastocysts. In addition, since maturation of lysosomes might be involved in the first step of cavity formation, we focused our attention on these organelles by means of the cytochemical localization of trimetaphosphatase activity and by the study of the effects of chloroquine on precavitation embryos. Our results suggest that cavitation starts in a few external cells (presumably competent cells entering the sixth cell cycle), by the chloroquine-sensitive formation of degradative autophagic vacuoles engulfing lipid droplets and vacuoles containing osmiophilic material. These complex structures enlarge (as a result of lipid metabolism?) and so transform into intrablastomeric cavities which, by means of a membrane fusion process, very rapidly become extracellular cavities that coalesce. The abembryonic pole of the blastocyst is determined in this way. Moreover, we suggest that the juxtacoelic cytoplasmic processes covering the inner cell mass (ICM) cells, which are known to restrict the expression of their totipotency during early cavitation (Fleming et al. 1984), are the latest remnants of the walls of the growing intrablastomeric cavities.

Key words: Lysosomes – Ultrastructure – Chloroquine – Blastocyst – Mouse

Introduction

Blastocyst formation is the first overt morphogenetic event in mammals. In the mouse, it normally takes place at the 32-cell stage (Smith and McLaren 1977; Alexandre 1979; Chisholm et al. 1985) as a consequence of a coordinate sequence of molecular and cytological events (Johnson et al. 1984). The latter are initiated at the 8-cell stage and consist of the close apposition of the blastomeres, termed compaction (Ducibella and Anderson 1975; Ducibella et al. 1977), together with cell polarization at both membrane (Mulnard and Huyghens 1978; Reeve and Ziomek 1981) and cytoplasmic levels (Ducibella et al. 1977; Reeve 1981). Intercellular junctions develop afterwards (Ducibella and Anderson 1975, 1977).

Cell polarity was shown to be required for the initial production of nascent blastocoele fluid (Wiley and Eglitis 1981; Wiley 1984), while compaction provides the conditions which allow the development of a large extracellular cavity during blastocyst growth (Johnson et al. 1979). Cavitation always starts at the periphery of the morula and thus seems to involve only a few cells marking the site of the abembryonic pole. Garbutt et al. (1987) have suggested that these cells might be the first to reach the 6th cell cycle, and thus determine the embryonic-abembryonic axis. The enlargement of the blastocoelic cavity has been reported to be caused by the passive entry of water, resulting from the Na^+/K^+-ATPase catalysed vectorial translocation of Na^+ into the cavity (Wiley 1984; Manejwala et al. 1986). This process seems to be controlled by an activatable adenylate cyclase which has been shown to stimulate sodium uptake from the external medium (Manejwala et al. 1986; Manejwala and Schultz 1989). However, the initial cytological event responsible for the eccentric blastocoele formation is still unknown.

It has been proposed that the early blastocoele might be derived from the coalescence of intercellular spaces that are formed just before cavitation (Calarco and Brown 1969). From a cytological point of view, it was suggested that both lipid droplets and mitochondria are responsible for the origin of the nascent blastocoele fluid (Wiley and Eglitis 1981). On the other hand, the ultrastructural study of Gianguzza and Mulnard (1972)

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showed that lysosomes might take part in the first steps of cavity formation. However, no experimental evidence has yet been produced to support this hypothesis. To address these questions, we undertook a careful ultrastructural study of nascent and growing blastocysts in vitro. In order to assess the role of lysosomes in the cavitation process, we treated precavitation-stage embryos with chloroquine, a lysosomotropic drug (de Duve et al. 1974) and analysed the lysosomal compartment by the cytochemical detection of trimetaphosphatase (TMP) activity at the ultrastructural level.

Materials and methods

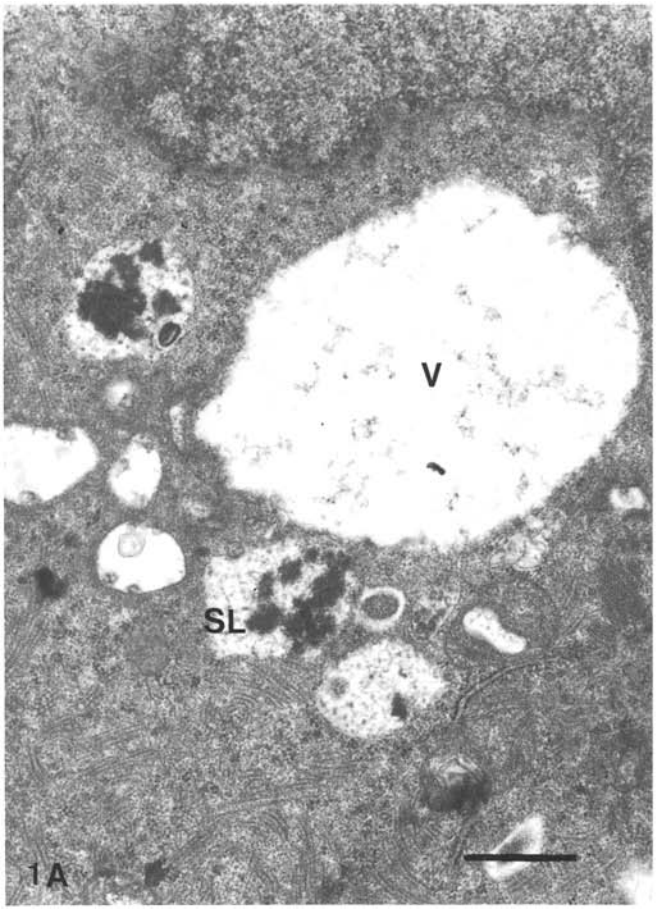
Embryos: collection, culture and treatment. Six to 10-week-old NMRI female mice were induced to superovulate by intraperitoneal injections of 5 IU pregnant mare's serum gonadotropin (PMSG) (Folligon, Intervet) at 17:00 h followed by 5 IU human chorionic gonadotropin (hCG) (Pregnyl, Organon) 48 h later. They were then caged with males overnight and inspected for vaginal plugs the following morning. Spontaneously ovulating females were also used for several experiments.

Two-cell stage embryos were collected by flushing the oviducts 46 h after the hCG injection (day 2 of pregnancy). Eight-cell-stage embryos were flushed from the uterine horns 64 h post-hCG (morning of day 3 of pregnancy).

Embryos were cultured at 37° C in 0.8 ml culture medium under 0.5 ml paraffin oil in Nunclon four-well multidishes, in a humidified atmosphere of 5% CO_2 in air. The culture medium consisted of a modified Krebs-Ringer bicarbonate (KRB-4; Mulnard and Puissant 1984), supplemented with 4 mg/ml crystallized and lyophilized bovine serum albumin (Sigma). Chloroquine (Sigma) was dissolved directly in the culture medium at a final concentration of 10 μM.

Morphology. Embryos were fixed in 2% glutaraldehyde in fresh 0.1 *M* cacodylate buffer (pH 7.3), with 5% sucrose, for 30 min at 4° C. Embryos were washed overnight in the same buffer, post-fixed in 1% OsO_4 for 1 h at 4° C, dehydrated in a graded ethanol series and embedded in Epon resin. Ultra-thin sections were contrast-stained with uranyl acetate for 20 min, followed by lead citrate for 10 min, and examined with an AEI EM 6B electron microscope at 60 kV.

Ultrastructural cytochemistry. Secondary lysosomes were localized using the histochemical procedure of Berg (1960), modified by Doty et al. (1977). Fixed and washed embryos were incubated with agitation at 37° C for 60–90 min in TMP medium prepared as follows: 18 mg sodium trimetaphosphate (Sigma) was dissolved in 4.5 ml of 0.1 *M* acetic acid; 50 ml distilled water and 10 ml 1.5% lead acetate were added and the pH was adjusted to 3.9 with 0.1 *M* nitric acid. The volume was brought to 100 ml with distilled water and 5 g sucrose added. Embryos were then rinsed thoroughly with distilled water, and treated with dilute ammonium sulphide to visualize the reaction product. They were finally post-fixed in 1% OsO_4, dehydrated and embedded in Epon resin. Ultra-



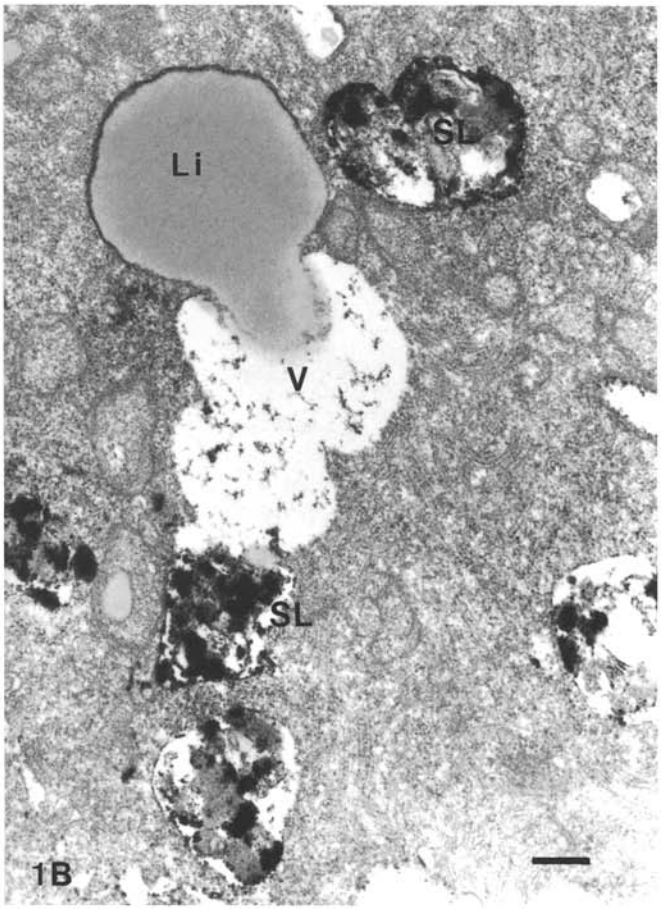
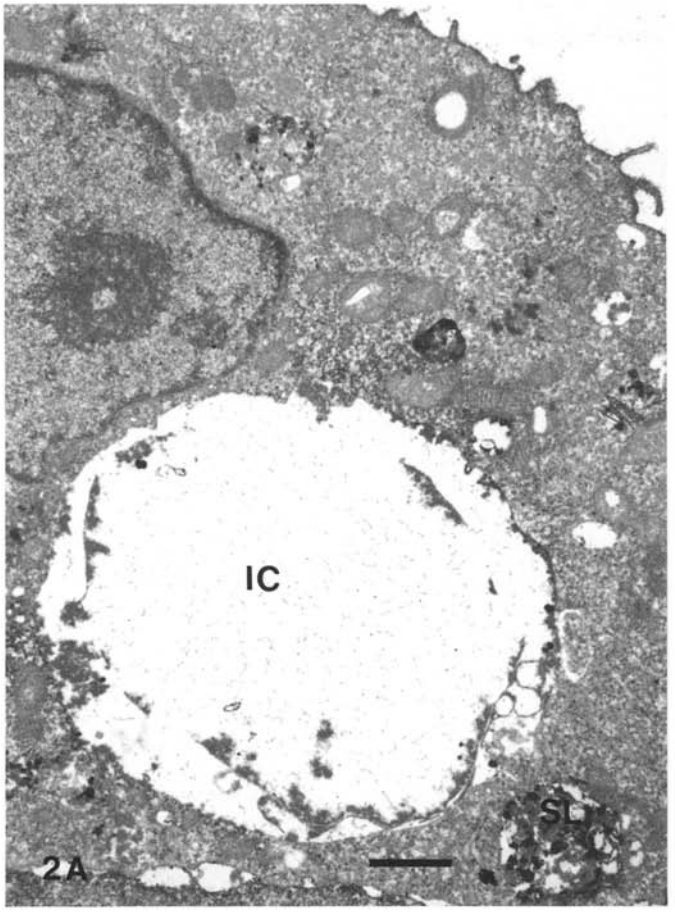


Fig. 1 A, B. Morulae. **A** Intrablastomeric vacuole (*V*) and secondary lysosomes (*SL*) in an external cell. **B** Complex structure made of a lipid droplet (*Li*), a vacuole (*V*) and a secondary lysosome (*SL*). Localization of trimetaphosphatase (TMP) activity. *Bars* = 0.5 μm



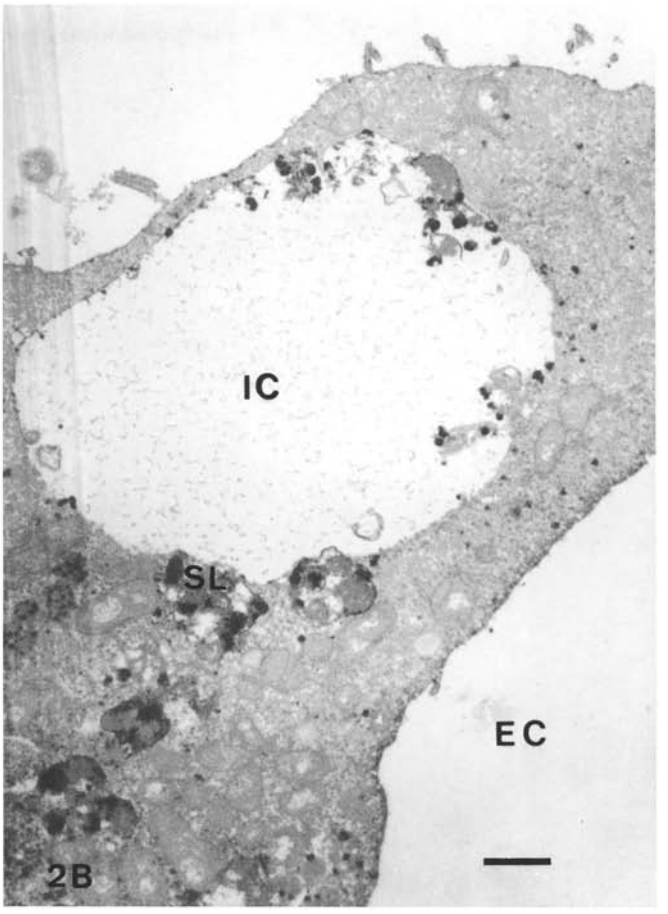


Fig. 2A, B. Nascent blastocysts (localization of TMP activity). **A** One single intrablastomeric cavity (*IC*). *SL*, Secondary lysosome. **B** External portion of an embryo displaying both an intracellular (*IC*) and an extracellular cavity (*EC*). *SL*, secondary lysosome. *Bars* = 1 μm

thin sections were lightly contrast-stained (5 min uranyl acetate and 5 min lead citrate). Berg's solution, in which sodium trimetaphosphate has been omitted, was used as a control.

Polysaccharides were detected in Epon thin sections by the method of Thiéry (1967). Embryos were fixed with 1.6% glutaraldehyde in 0.1 *M* Sörensen phosphate buffer (pH 7.4) for 30 min at 4° C, washed overnight in the same buffer and post-fixed in 1% OsO_4 or 30 min at 4° C. After oxidation with periodic acid, sections were treated for 20 h with thiocarbohydrazide made visible with silver proteinate.

Results

Blastocoele formation and expansion

According to the well-documented asynchrony in development (Alexandre 1979), samples of embryos cultured for 48–55 h from the 2-cell stage onward consist of a mixed population of morulae, nascent blastocysts and growing blastocysts. Morulae and growing blastocysts were immediately selected and fixed separately. Nascent blastocysts were chosen by examining embryos at 90-min intervals with a Wild M20 dissecting microscope and selecting for the first appearance of a small blastocoelic cavity, as previously described (Alexandre 1979; Fleming et al. 1984).

Morulae. In agreement with previous reports (Fleming et al. 1984), secondary lysosomes were more abundant in the presumptive trophectoderm cells (Fig. 7) and were localized in the basal regions. They were found together with vacuoles and lipid droplets. In a few of these external cells, vacuoles containing osmiophilic material were found in the close vicinity of secondary lysosomes (Fig. 1A). We also observed complex structures consisting of associated secondary lysosomes, vacuoles and lipid droplets (Fig. 1B). Both structures are rarely observed in late morulae, and the latter are never found in earlier stages. On the other hand, TMP-positive material is found associated with both secondary lysosomes and complex structures (Fig. 1B).

Nascent blastocysts. Fifteen nascent blastocysts were analysed. Only one displayed a single intracellular cavity in an external blastomere and no extracellular cavity (Fig. 2A). In 6 other nascent blastocysts, both intra- and extrablastomeric cavities were detected (Fig. 2B). In the last 8 embryos, only extracellular cavities were observed. Intracellular cavities, in contrast to extracellular ones, are characterized by the presence of numerous polygranules in their lumen and TMP-positive material at their edges. The intrablastomeric cavities are closely associated with secondary lysosomes which are still more

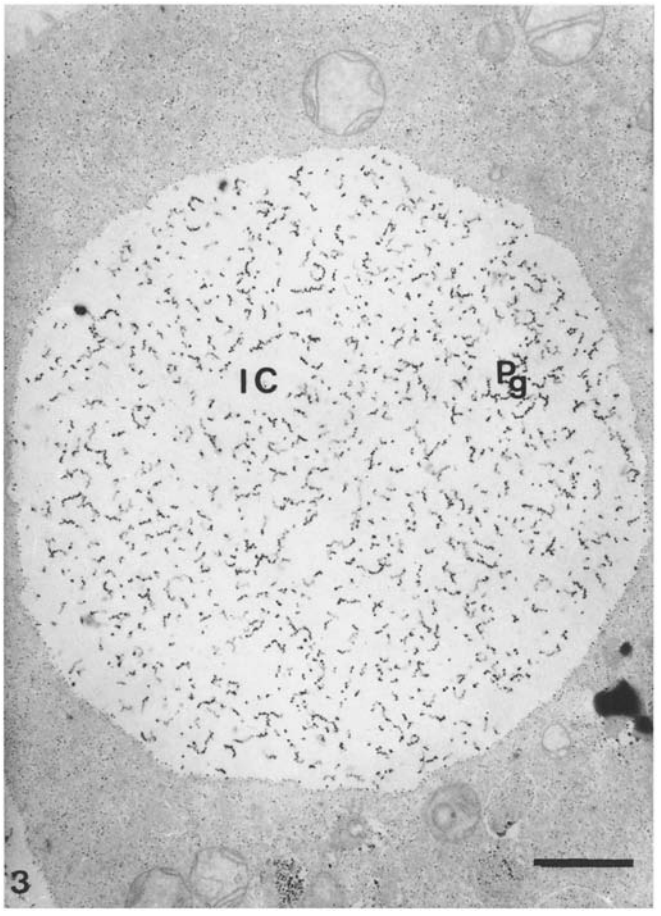


Fig. 3. Nascent blastocyst. Intracellular cavity (*IC*) with polysaccharidic polygranules (*Pg*); Thièry's staining method. *Bar* = 1 μm

abundant in external than in internal cells (Fig. 7). Lipid droplets are much less abundant than in morulae and are often found integrated into complex vesicles like the ones shown in Fig. 1B. The Thiéry staining procedure was specific for the luminal polygranules (Fig. 3), which demonstrates their polysaccharidic nature.

Growing blastocysts. Cross-sections perpendicular to the embryonic-abembryonic axis of early growing blastocysts revealed the presence of two or three extracellular cavities fusing together at the abembryonic pole. Cytoplasmic pillars between the cavities are enriched with secondary lysosomes positively stained for TMP (Fig. 4A, B). At this stage, vacuolar structures and large intracellular cavities are no longer found. Lysosomes are still visible in the cytoplasmic processes of trophectodermal cells, lining the inner cell mass (ICM) of the growing blastocysts, at a slightly later stage (Fig. 4C).

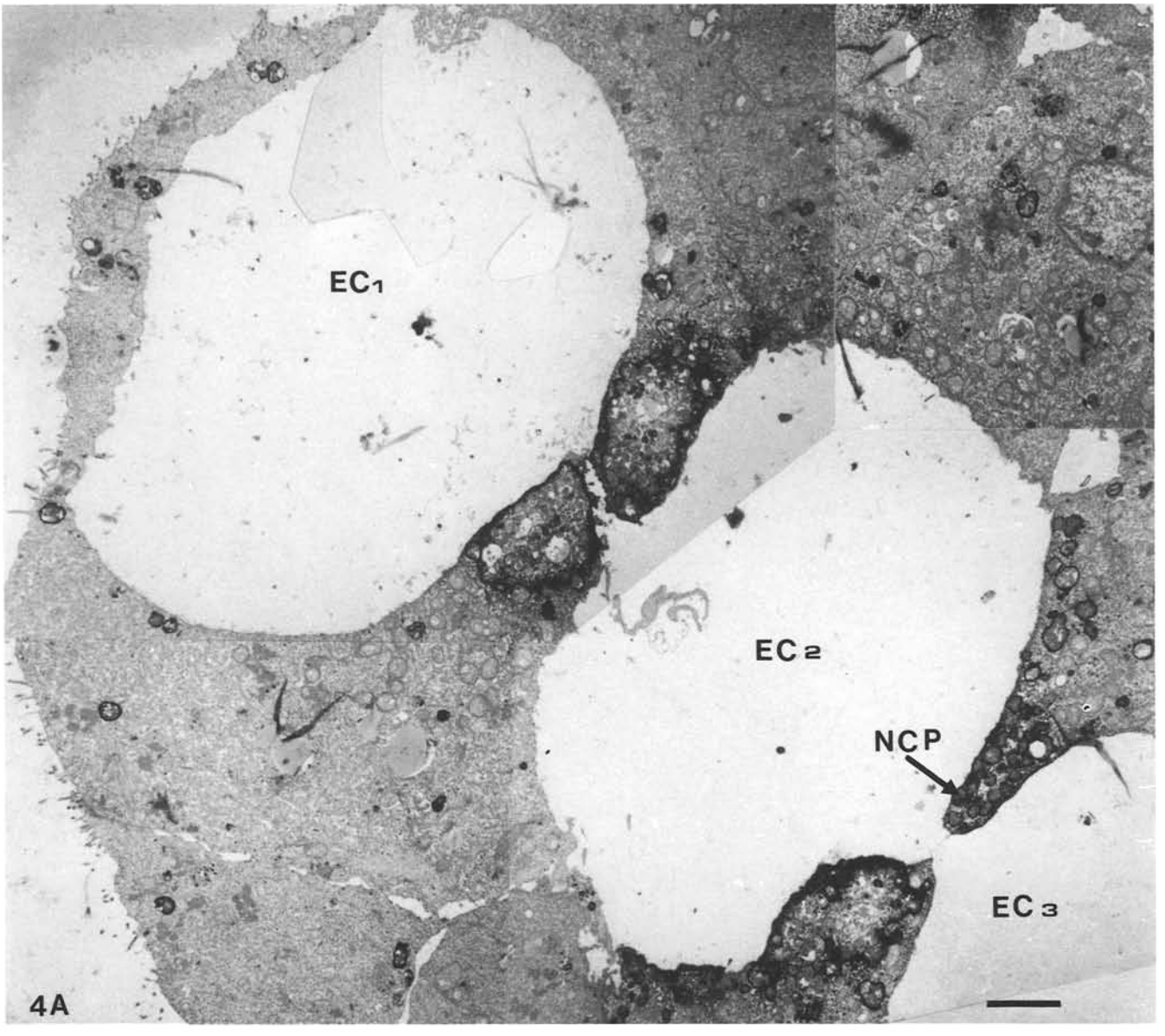
Effect of chloroquine on cavitation

Since our ultrastructural observations strongly suggested a central role for lysosomes in the initiation of cavitation, we treated late morulae with 10 μ*M* chloroquine and examined them at 90-min intervals for the appearance of the blastocoelic cavity. Precavitation embryos were treated according to their presumptive commitment to undergo cavitation. We therefore selected the "non-cavitating" embryos from populations in which 5–10% (Fig. 5A) or 25–40% (Fig. 5B) were nascent or growing blastocysts, assuming that the latter are more advanced in their commitment to cavitation. Indeed, it appears from Fig. 5 that the two curves for untreated embryos display an obvious difference in their slope, in agreement with the well-documented sigmoid pattern of the cavitation curve for normal embryos (Garbutt et al. 1987). In both experimental groups, however, cavitation proceeds for the first 4.5 h in the presence of 10 μ*M* chloroquine, but is subsequently inhibited. In addition, ultrastructural analysis of morulae prevented from undergoing cavitation shows that secondary lysosomes are accumulated within the cytoplasm of the outer blastomeres, as seen in Fig. 6. The number of cross-sectioned secondary lysosomes was counted on low-magnification micrographs of whole cells only. The values reduced to the arbitrary surface of 200 μm^2 are given in Fig. 7. The clearcut accumulation of lysosomes was observed in both internal and external blastomeres. No other subcellular alterations were found.

Discussion

The considerable developmental heterogeneity among expanding blastocysts results from both differences in the timing of ovulation and/or fertilization of oocytes in vivo (Bolton et al. 1984; Howlett and Bolton 1985), and from variability in the length of the cell cycles (Chisholm et al. 1985). This makes the study of the onset of cavitation quite difficult. To overcome this difficulty, embryos have usually been examined at regular intervals and collected as they commenced cavitation (Alexandre 1979; Chisholm et al. 1985). We have shown in this study that most of these "Oh-blastocysts" display an extracellular cavity. This explains why previous ultrastructural studies have led to the conclusion that the first accumulation of blastocoelic fluid takes place within pre-existing intercellular spaces (Calarco and Brown 1969). According to this proposal, the nascent blastocoele has an extracellular origin, but the involvement of cellular organelles in blastocoele formation has also been widely suggested. Cytoplasmic vesicles (or doplets) of unknown origin were shown to increase in both number and size (from 1 to 3 μm in diameter) from fertilization onwards, and to disappear at cavitation (Calarco and Brown 1969). Since they have been shown to undergo a microtubule,

Fig. 4A–C. Growing blastocysts (localization of TMP activity). **A** Tangential section through the forming abembryonic pole showing the coalescence of three extracellular cavities (*EC1-3*). *NCP*, Necrotic cytoplasmic processes filled with secondary lysosomes. **B** Enlargement of two apposed necrotic cytoplasmic processes. *EC1-2*, Extracellular cavities 1 and 2. **C** Palely stained trophectodermal cell (*Tr*) with a juxtacoelic process containing numerous secondary lysosomes (*SL*). *Bars* = 2 μm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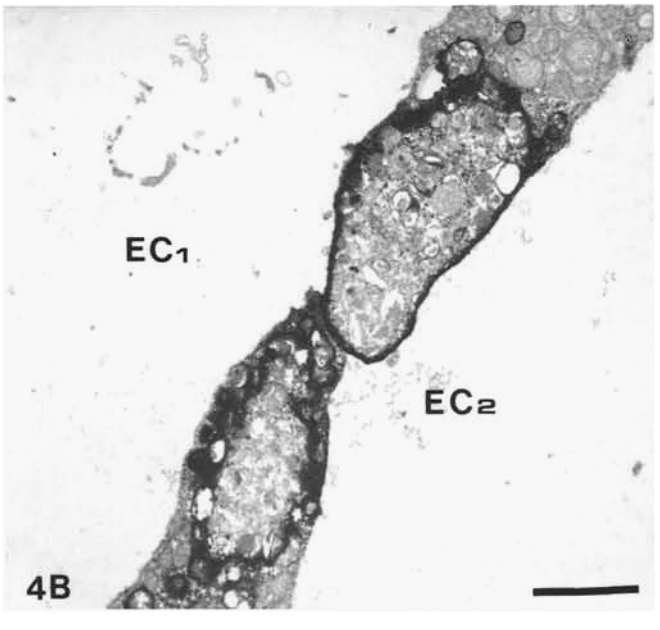
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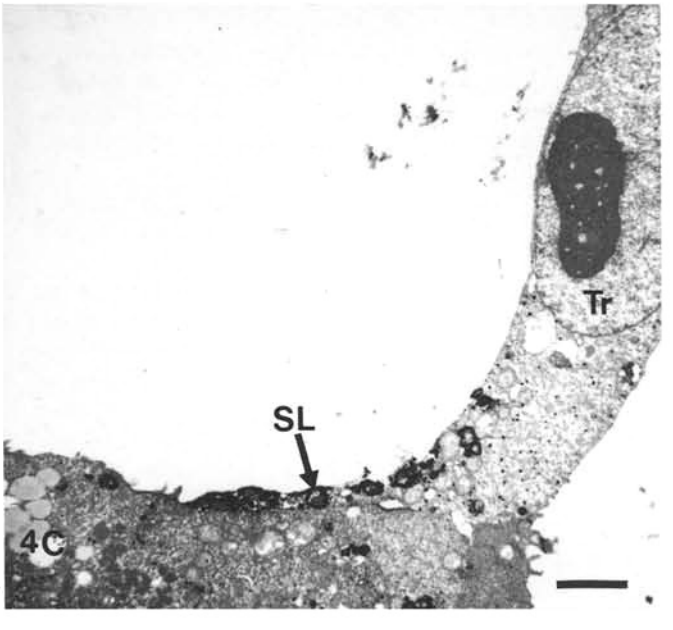
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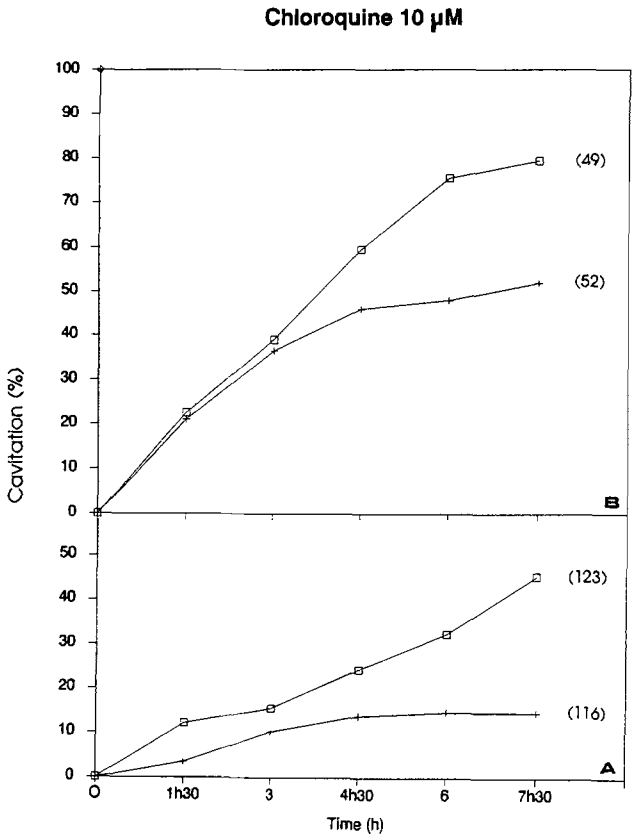

Fig. 5A, B. Effect of 10 µ*M* chloroquine on blastocyst formation according to the duration of treatment. No cavitating embryos were isolated from populations in which 5–10% (**A**) or 25–40% (**B**) had cavitated. □, Untreated embryos; +, treated embryos. Total number of embryos in *parentheses*

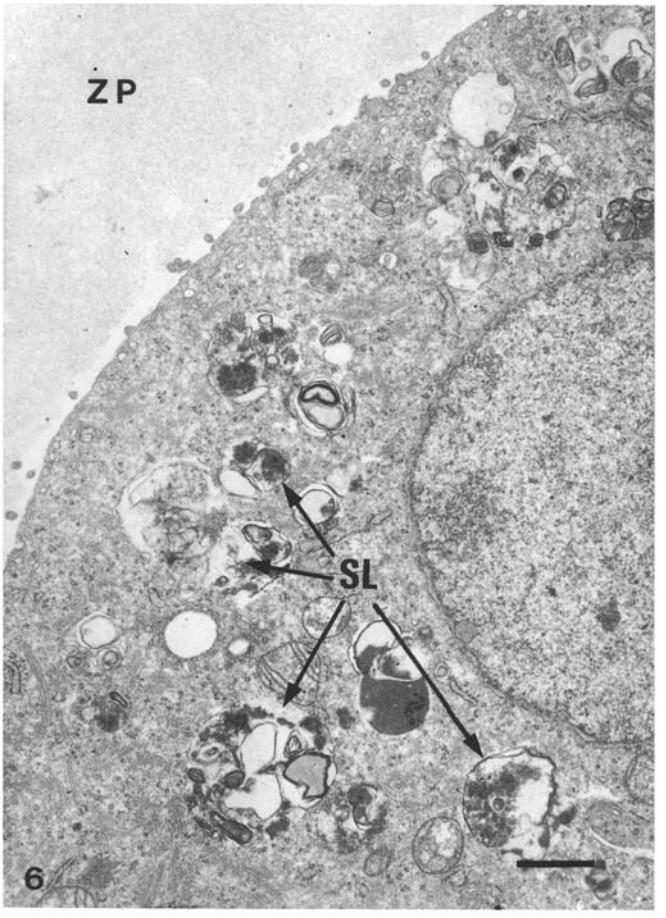

Fig. 6. Outer blastomere of a morula treated for 6 h with 10 µ*M* chloroquine showing an accumulation of secondary lysosomes (*SL*). *ZP*, zona pellucida. *Bar* = 1 µm

microfilament and Ca^{2+}-dependent relocation under the contiguous cell surfaces soon before cavitation (Ducibella and Anderson 1975; Wiley and Eglitis 1980, 1981), it has been suggested that one of the cellular processes mediating cavitation involves vesicle (droplet)-mediated secretion. On the other hand, the co-localization of droplets and mitochondria allowed Wiley and Eglitis (1981) to speculate that, if the droplets contain lipids, the cortical localization of the two structures provides an efficient means of positioning an intra-embryonic source of nascent blastocoele fluid, or ATP, which is needed for the metabolic conversion of lipid to water, on the one hand, and its substrate, on the other.

Secondary lysosomes are also known to undergo a polarization in the basal cytoplasm of external blastomeres from the 16-cell stage onwards (Fleming and Pickering 1985; Maro et al. 1985). A few ultrastructural and cytochemical studies have suggested that lysosomal activity could display a morphogenetic function during blastocoele formation, taking part in the first steps of cavity hollowing. A polymorphic lysosomal system is indeed observed in mouse cleaving embryos from the 8-cell stage onwards, and expands particularly in morulae, under both in vivo and in vitro conditions (Gianguzza and Mulnard 1972; Liu Bin 1981; Geuskens and Alexandre 1982). We describe here, for the first time, unusual "complex structures", seemingly resulting from

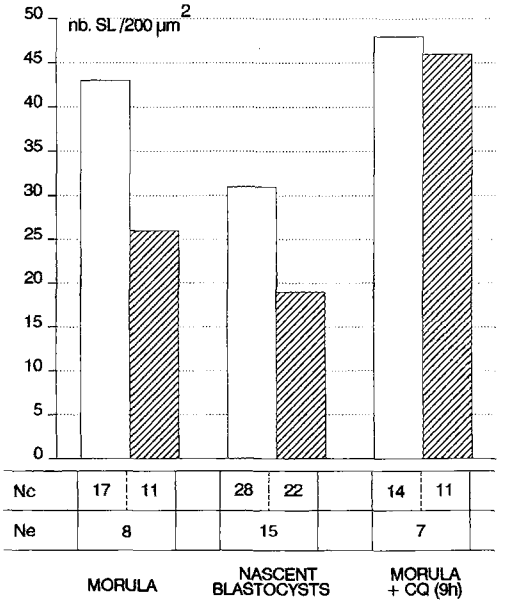

Fig. 7. Number of secondary lysosomes per 200 µm² in morulae, nascent blastocysts and "non-cavitating" morulae treated with 10 µ*M* chloroquine for 9 h. *Nc*, Number of cells analysed; *Ne*, number of embryos analysed. □ External cells; ▨ Internal cells

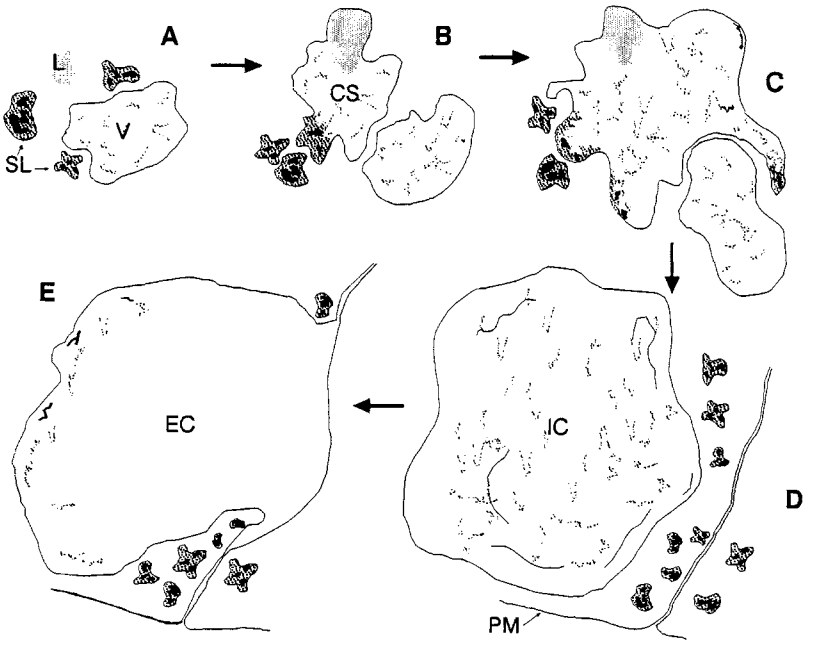

Fig. 8 A–E. Diagrammatic representation of the hypothetical sequence for the early extracellular blastocoele formation in the cleaving mouse embryo. The **A** to **B** transition is thought to be the chloroquine-sensitive step. *SL*, Secondary lysosomes; *L*, lipid droplet; *V*, cytoplasmic vesicle; *CS*, "complex structure"; *IC*, intracellular cavity; *EC*, early extracellular cavity; *PM*, plasma membrane. All the organelles are described in the text

the coalescence of lipid droplets, granular vesicles and secondary lysosomes. They are obviously very transitory structures, since they are only found in late morulae and very early nascent blastocysts. They may correspond to the "degradative autophagic vacuoles" recently described by Dunn (1990a, b) in his model of the autophagic vacuole formation and maturation. Interestingly, Bieliavsky and Geuskens (1990) have recently described similar structures in their study of the sequence of vesicle transformation, leading to the formation and completion of interblastomeric membranes in *Xenopus laevis* embryos. The structures result from the integration of lipid droplets by vesicles which subsequently become large glycogen-containing vacuoles.

Large intracellular vacuoles are other very transitory structures found slightly later in only a few external cells in Oh-blastocysts (Fig. 2A). They also display several features of autophagic vacuoles, such as internal membrane debris and acid hydrolase acquisition (Holtzman 1989), as revealed by the presence of the TMP-positive material near their limiting membrane. In addition, they contain osmiophilic material, as do the early small vesicles. Our cytochemical analysis clearly demonstrates that this material is made up of polysaccharides (glycogen), which is thus a useful marker for intracellular vacuoles. Taken together, these observations suggest that the "complex structures" may be the very early nucleating centres for the larger intracellular vacuoles.

The presence in some early extracellular cavities of similar osmiophilic (polysaccharidic) material (data not shown; Geuskens and Alexandre 1982) suggests that the fast-growing primary intracellular cavities suddenly become external. One can easily imagine this transition as a result of a membrane fusion process resembling exocytosis, as shown in Fig. 8.

This hypothesis thus proposes, for the first time, that development of transitory intracellular cavities is a normal step of cavitation. Large intracellular cavities are, however, known to occur under experimental conditions. Embryos incubated in an antiserum which reversibly inhibits compaction, with no effect on cell division, result in a high incidence of aggregates of fluid-accumulating cells containing vacuolar structures (Johnson et al. 1979). Morulae treated with demecolcine also display intracellular cavities restricted to a few external cells (Wiley and Eglitis 1980). Two- or 4-cell mouse embryos, released from cytochalasin D arrest, also develop a large intracellular vacuole (Kimber and Surani 1981). Finally, one single large intrablastomeric vacuole containing osmiophilic material has been described previously in one embryo treated for 39 h with methylglyoxal-*bis*-guanylhydrazone, an inhibitor of polyamine biosynthesis (Geuskens and Alexandre 1982). In these four examples, vacuolization may have been an exaggerated form of a normal process.

Since cavitation always starts at the periphery of the morula, our hypothesis might explain how the abembryonic pole of the blastocyst is determined. Indeed, it has been demonstrated that only embryos in which cells have entered the 6th cell cycle will form a blastocoele (Smith and McLaren 1977; Braude 1979), and that the first cells to reach the 6th cell cycle are external and are the ones which acquire competence to form a blastocoele (Chisholm et al. 1985; Garbutt et al. 1987). We suggest that these cells actually achieve competence to undergo a lysosome-mediated intracellular hollowing. The use of chloroquine indicates that this transient morphogenetic event takes place during a very short temporal window, at about 4.5 h before the appearance of a true blastocoele. This model is in total agreement with the hypothesis that the first cells to reach the 6th cell cycle would mark the abembryonic pole of the embryo (Garbutt et al. 1987).

This study has also shown that when two or three separated extracellular cavities are generated, their rapid coalescence is achieved by retraction of the mural cyto-

plasmic processes of the neighbouring cells, which involves a lysosome-mediated morphogenetic necrotic process, as seen in Fig. 4. Such a necrotic process also seems to be responsible for the retraction of the juxtacoelic processes of the same external cells that takes place at least 12 h later. These processes, which form a continuous layer separating the ICM cells and the blastocoelic fluid (Ducibella et al. 1975), were actually shown to perform a fundamental role, that of restricting the expression of totipotency of ICM cells during early cavitation (Fleming et al. 1984). At that stage, only those cells surrounding the nascent blastocoele are fluid-transporting cells, since they contain diluted cytosol and stain palely (Fleming et al. 1984; Fig. 4C).

In conclusion, our ultrastructural analysis, together with the study of the effect of chloroquine [a drug known to cause a pronounced elevation of intralysosomal pH and thus to block the activity of acidic hydrolases (Wibo et al. 1974), allows us to propose a sequence of cytological events for the polarized blastocoele formation in the cleaving mouse embryo. It is schematically summarized in Fig. 8. A lysosome-mediated intracellular hollowing takes place in the few external cells which first reach the 6th cell cycle. This early chloroquine-sensitive step is followed by a rapid enlargement of the intracellular cavities, which results from both lipid metabolism and active pumping of water, as suggested by the presence, of Na^+-K^+-ATPase on their membranes (Aziz and Alexandre, unpublished data). An exocytic process very rapidly transforms these growing intracellular cavities into a large extracellular cavity which will enlarge as previously described (Wiley 1984; Manejwala and Schultz 1989).

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