‘Blebbing’ of the nuclear envelope of mouse zygotes, early embryos and hybrid cells

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Summary

In the mouse zygote and in two-cell stage embryos the inner leaflet of the nuclear envelope of pronuclei and that of polar body II nuclei evaginate, forming multiple blebs within the perinuclear space, which contains a granular material. Blebbing exists only in oocytes activated by sperm in vivo or in vitro, or parthenogenetically by treatment with ethanol or puromycin. The germinal vesicle and an interphase nucleus formed after treatment of the oocyte at metaphase I by puromycin do not form blebs. Formation of blebs is specifically located in the cell cycle. The burst of the blebbing activity occurs during the first half of the cell cycle in one-cell embryos and in the earliest interphase period in the second cell cycle. Blebbing ceases from the beginning of the third cell cycle. The occurrence in the cytoplasm of ‘double-layered’ vesicles containing granular material resembling bleb contents and the disappearance of blebs from the nuclear envelope by the end of the cell cycle provide evidence that blebs represent a step in the transport of some material from the nucleus to the cytoplasm. Ethanolic phosphotungstic acid does not stain blebs, suggesting the absence of basic protein in their contents.

Blebbing can be induced in somatic (thymocyte) and embryonic (blastomere of 8-cell stage embryo) nuclei following cell hybridization with activated oocytes. Their response to the oocyte cytoplasm by initiating blebbing depends on: (1) the position of the host cell in its cell cycle at the moment of hybridization, and (2) the time spent by the foreign nuclei in the host cytoplasm following cell fusion. If donor nuclei are introduced close to the time of activation, they start to produce blebs at the time corresponding to the initiation of blebbing by the female pronucleus in the first cell cycle. If foreign nuclei are introduced a few hours after activation they must be incubated in the host cytoplasm for some time before initiation of bleb formation, provided that the host pronucleus has initiated blebbing by that time.

The existence of blebbing in nuclei formed only after oocyte activation, and the timing and the general occurrence of this event during the earliest cleavage stages of almost every mammalian embryo, suggest that this special nucleocytoplasmic transport plays a specific role at the beginning of development.

Key words: blebs, nuclear envelope, early mouse embryo, cell hybrids.

Introduction

In almost all mammalian zygotes studied by electron microscopy, small evaginations of the inner leaflet of the nuclear envelope (NE) have been observed in both pronuclei, which project into the perinuclear space. Evaginations of various sizes containing an electron-dense, non-homogeneous material have been referred to in the literature as tertiary nucleoli (Szollósi, 1965), blebs (Gondos & Bhiraleus, 1970; Gulyas, 1971a; Longo, 1978) and nuclear extrusions (Gulyas, 1971b). In the present work we will use the term ‘bleb’ and ‘blebbing’ as the most descriptive, most neutral and most commonly used term in the literature.

Nuclear envelope bleb formation was described for the first time in rat pronuclei by Szollósi (1965) and later in rabbit zygotes by Gulyas (1971b). In the rat bleb formation ceases at the end of the first cell cycle, but in the rabbit the blebbing process has been observed not only in pronucleate eggs (Zamboni & Mastroianni, 1966; Gondos & Bhiraleus, 1970; Gulyas, 1971a) but also in two-cell (Gulyas, 1971b; Longo, 1978) and four-cell embryos (D. Szollósi, unpublished data). Blebs have been described also in rabbit blastocysts by Hadek & Swift (1962). Among other mammals in which nuclear envelope blebs have been detected in zygotes are: sheep...
unpublished). In the laboratory mouse, blebbing has a limited duration because blebs have never been seen in morulae or in blastocysts (M. Szollosi, unpublished). In the human, blebs have been described in pronucleate eggs (Zamboni et al. 1986; Santhananthan & Trounson, et al. 1983). However, blebbing was never observed either in oocytes or in any early embryonic stages of the domestic pig (Szollosi & Hunter, 1973; D. Szollosi, unpublished). Nuclear blebbing has also been described in oocytes from foetal and prepubertal ovaries in cattle, rhesus monkeys and humans (Baker & Franchi, 1969), but they have never been seen in nuclei (germinal vesicles) of ovarian oocytes from the large antral follicles of adult females of rabbit, mouse, pig, sheep and humans (D. Szollosi, unpublished).

The above information strongly suggests that blebbing of the nuclear envelope is a common event during early mammalian development. Surprisingly, this phenomenon is known from fragmentary studies only, and its biological significance is at present far from being understood. Because fertilization and early embryonic development of the laboratory mouse can be easily controlled and experimentally investigated we carried out a detailed study on the dynamics of blebbing shown by the nuclear envelope in this species.

Materials and methods

Ovarian oocytes

Swiss albino female mice (8–10 weeks old) were treated by an intraperitoneal injection of 5 i.u. of pregnant mare's serum gonadotrophin (PMSG) for development of ovarian follicles. Oocytes were removed, 48 h after PMSG injection, from ovarian follicles with a sharp sterile needle in medium 2 (Quinn et al. 1982) containing dibutyryl cyclic AMP (100 μg ml⁻¹) and fixed for electron microscopy (EM).

Zygote and embryos

Ovulation was induced in female mice of CBA-T676T6 and CBA-H strains by intraperitoneal injections of PMSG and human chorionic gonadotrophin (hCG; 5–10 i.u. of each), at 48-h intervals, and females were mated with males of their own strain. Fertilized eggs were flushed from the oviduct at almost hourly intervals between 16 and 34 h after hCG administration, and two-cell stage embryos were removed at 32, 34, 39, 40, 50, 56 h after hCG, three- to four-cell embryos at 50, 56 h, and six- to eight-cell embryos at 66 h after hCG. Embryos were fixed immediately for EM.

Puromycin treatment

Oocytes were collected from female OF1 treated with hormones. The cumulus was removed with hyaluronidase (300 i.u. ml⁻¹ of medium M2), and the zona pellucida with α-chymotrypsin (30 μg ml⁻¹ of medium M2). Sperm for fertilization in vitro were taken from the cauda epididymis of adult F1 (C57 × CBA) males, and capacitated for 1-5 h at 37-5°C in Whittingham's medium for fertilization in vitro (FIV) (Fraser & Drury, 1975). Puromycin was used at a concentration of 100 μg ml⁻¹ of culture medium. All culture was carried out under paraffin oil.

Ovarian oocytes were collected from ovaries 48 h after injection of PMSG. They were cultured in medium M16 (Whittingham, 1971) for 9-5 h. Oocytes in metaphase I stage were transferred to medium M16 containing puromycin for 12 h of culture. Oocytes that developed nuclei and extruded PB1 were fixed for EM.

Ovulated oocytes were collected 16 h after hCG injection, and after removal of follicular cells were divided into four groups: (1) oocytes cultured for 9 h in M16 containing puromycin; oocytes that developed pronuclei, i.e. were activated by drugs, were fixed for EM; (2) zona-free oocytes inseminated in medium for 2 h containing puromycin and then transferred to M16 with puromycin for 7 h of culture; (3) zona-free oocytes inseminated and cultured as for group (2), but both media contained tritiated methionine at a final concentration of 20 μCi ml⁻¹ in addition to puromycin; (4) zona-free oocytes inseminated and cultured as for group (2) but both media contained triitated methionine and no puromycin, in order to control the influence of puromycin on methionine incorporation. Fertilized oocytes were fixed for EM.

Autoradiography

Semithin sections of oocytes from groups (3) and (4) were placed on slides and dried. They were coated with emulsion (Ilford K-5, stored for 6 days in a light-proof box at 4°C) then developed in D-19 (Eastman Kodak) or Whitten's medium (Whitten, 1971) or in medium 16 under liquid paraffin and cultured for 0-5-2-5 h (37°C, 5 % CO₂ in air) before handling.

Thymocytes were obtained from thymus of 1- to 5-day-old newborn mice as described by Czolowska et al. (1984). Blastomeres were isolated from eight-cell mouse embryos of Swiss albino females mated with F1 (C57 BL/10 × CBA) males on the third day of pregnancy. A few embryos with less than eight cells were always present among the uncompacted eight-cell embryos. Thus they were considered to be embryos from the first half of the four-cell cycle.

Oocytes, either before or after cell hybridization, were activated with 7-8 % ethanol in culture medium for 5 min at room temperature under liquid paraffin, according to the method of Cuthbertson (Cuthbertson et al. 1981; Cuthbertson, 1983).

Cell fusion was achieved by agglutinating thymocytes or a blastomere to an oocyte with phytohaemagglutinin and treating agglutinated cells with polyethylene glycol (PEG: Loba-Chemie M, 1000 50 % (w/v)) and Fluka (M, 2000 45 % (w/v)). PEG-treated cells were thoroughly washed and then cultured for various lengths of time according to the standard procedure, as given by Czolowska et al. (1984).

Three groups of hybrid cells were produced: (1) oocyte-thymocyte hybrids, which were parthenogenetically activated 20-45 min after fusion with PEG and cultured for 2-5 h, 6-7 h and 17 h after activation; (2) oocyte-thymocyte hybrids, which were formed by fusion of thymocytes with oocytes that had been activated and cultured 5-6 h before fusion. Hybrids
were cultured for 1–2.5 h; (3) oocyte–blastomere hybrids were produced at 1 h, 2 h, 4.5 h after oocyte activation and cultured subsequently for 1–7 h. (Hybrids were prepared by Dr R. Czołowska, University of Warsaw.)

Electron microscopy
Oocytes, embryos and hybrid cells were fixed for 45–60 min in a mixture of 2.5 % glutaraldehyde, 0.7 % paraformaldehyde, 0.075 M-phosphate buffer, pH 7.2–7.4, containing 0.2–0.5 % potassium ferricyanide (Yotsuyanagi & Szollosi, 1981). They were then washed in the same buffer, osmicated in 2 % OsO₄ in distilled water, rinsed three times in distilled water and stained overnight in 0.5 % aqueous uranyl acetate solution. They were dehydrated in an ethanol series and embedded in Epon. Sections were stained with uranyl acetate and lead nitrate.

Phosphotungstic acid staining
Ovulated, fertilized in vitro oocytes were collected 21 h after hCG injection, fixed in 2.5 % glutaraldehyde in phosphate buffer, dehydrated and stained in 1 % phosphotungstic acid in absolute ethanol overnight (18 h) and embedded in Epon. Unstained sections were observed in the EM.

Bleb counting
Blebs were counted in zygote pronuclei, in nuclei of the second polar body and in nuclei of blastomeres on electron micrographs of sectioned specimens. Counts were made on the nuclear envelope along a distance equal to 10 μm, using photographs from three grids per specimen and from three specimens in every age group of embryos.

In hybrids and puromycin-treated oocytes the blebbing rate was not determined.

Results

Ovarian oocytes
The nuclear envelope (NE) of the oocyte nucleus (germinal vesicle, GV) corresponds to the classical descriptions of the NE. Its outline may be undulating and occasionally deeply folded. Local irregularities may be present but neither evaginations of the inner nuclear membrane (blebs) nor continuity of the outer leaflet with the endoplasmic reticulum has ever been observed. Polysomes are attached in place to the NE, while polysome-studded cytoplasmic vesicles are quite frequent (Fig. 1).

Fertilized eggs
The times indicated for the first and second cleavage divisions were approximate. In each group of embryos recovered we found either one- and two-, or two-, three- and four-cell stages, respectively. The sum of such observations permitted us to estimate that the first segmentation division took place approximately 31–34 h after hCG administration, and the second between 50 and 56 h. We arbitrarily divided each of the cell cycles into three periods: (1) early period: telophase and early interphase nucleus; (2) middle period: defined by half the hours required to complete the cycle; (3) late period: shortly before initiation of mitosis.

Embryos composed of four to eight blastomeres were collected from oviducts 66 h post hCG.

First cell cycle
Early period. NE formation proceeded more or less synchronously around both of the denuded gamete chromatin groups that formed the pronuclei 16–17 h after hCG (approx. 4–5 h after sperm penetration). Single, flattened smooth endoplasmic reticulum (ER) vesicles adhered to the surface of the chromatin in various regions. At this time nuclear pores could not be identified regularly. In the second polar body (PB II) NE formation lagged behind that in the pronuclei by 1–2 h. At 18–19 h after hCG injection all three types of nuclei, female, male and PB II nuclei, had complete NEs. Ribosomes were rarely attached to the NE of pronuclei.

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Fig. 1. Nuclear envelope (ne) of a mouse germinal vesicle (gv). Many nuclear pores are present. The width of the perinuclear space (ps) is uneven. Polysomes are attached to the cytoplasmic surface of the outer leaflet of the envelope and are also present on flattened cytoplasmic membrane rough endoplasmic reticulum (rer) vesicles. Chromatin lies under the NE on its nucleoplasmic side. ×45,000. Bar, 1 μm.

Fig. 2. The NE is formed at about 5 h after sperm penetration and both pronuclei form blebs (b) with granular contents that evaginate from the nucleus into the ps. They form half-circles and are limited by the inner leaflet of the NE. ×62,800. Bar, 0.25 μm.
Local NE surface irregularities, formed by enlargement of the perinuclear space, were observed in all nuclei. Granular electron-dense material adhered to the inner leaflet of the NE in small areas, which at these points formed small evaginations opening towards the nucleoplasm (Fig. 2). We shall refer to these evaginations as blebs. The forming blebs were identical in all three types of nuclei, only their numbers differed. The first blebs to form were semicircular in section, having a diameter of 60-160 nm. The blebs soon became circular, oval or pear-shaped with longer axes, up to 250 nm. The membrane surrounding the bleb was, during its formation, in continuity with the inner leaflet of the NE and as the bleb became larger a row of granules aligned forming a diaphragm-like structure at the level of the inner membrane (Figs 3–5). It sometimes resembled a pore complex (Unwin & Milligan, 1982) but the similarity seems to be superficial. Granules of the same size were also seen within the bleb contents. Occasionally the inner membrane of the NE appears to circumscribe the bleb (Fig. 4). The perinuclear space often enlarges extensively when two or more blebs develop close together (Fig. 3).
Table 1. Blebbing of the nuclear envelope in mouse zygotes and embryos

<table>
<thead>
<tr>
<th>Sub-cycle periods</th>
<th>Early</th>
<th>Middle</th>
<th>Late</th>
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<tr>
<td>Cell cycle</td>
<td></td>
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<tr>
<td>First</td>
<td>0.8 (0.2)</td>
<td>0.5 (rare)</td>
<td>0.07 (rare)</td>
</tr>
<tr>
<td>Second</td>
<td>0.8 (rare)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Third</td>
<td>Rare</td>
<td>n.i.</td>
<td>n.i.</td>
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<tr>
<td>Fourth</td>
<td>None</td>
<td>n.i.</td>
<td>n.i.</td>
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Values in table are mean no. of blebs per NE distance equal to 10 µm.

Blebbing of the NE in the second polar body nucleus given in parenthesis. n.i., not investigated.

No relationship exists between the number and distribution of nuclear pores and blebs (Figs 2, 5): NE segments with many pores and many blebs, those with many blebs and few pores, and the converse with few blebs and many pores, may be seen in different sections or different segments of the NE of the same nucleus. Blebbing activity was most intense during the early period of the first cell cycle, 18–20 h after hCG (Table 1). The number of blebs was almost the same in both pronuclei but is significantly lower in the PB II nucleus.

*Middle period.* At 25–26 h after hCG, the outer leaflet of the NE often developed into long, tubular outpocketings in direct spatial relationship with an enlarged perinuclear space containing blebs. The blebs may be still open or already closed at their base (Fig. 6). The tubular outpocketings were identical in sections to smooth tubular ER channels, found frequently around the pronuclei. In the proximity of NE smooth membrane vesicles containing structures identical to blebs were occasionally visible. They have similar granular content surrounded by a tight-fitting membrane (Fig. 7). In the middle period fewer blebs were seen than in the early period and blebs were only sporadically visible in the PB II nucleus (Table 1).

*Late period.* In the last period of the cell cycle, 28–34 h after hCG blebs were very rarely encountered along the NE of pronuclei, and were extremely rare or absent in the PB II nucleus (Table 1). Large vesicles were apposed occasionally along the inner component of the NE. Within these vesicles, structures similar to blebs are located but are less electron-dense (Fig. 8). IAL (intranuclear annulate lamellae) were occasionally present in pronuclei of the mouse.

*Second cell cycle*

*Early period.* At 32–34 h after hCG the reconstituted NE in both blastomeres had many pores and many blebs. Their mean number was comparable to the number given for the early period of the first cell cycle (Table 1). Small elevations of the external leaflet of the NE, forming enlarged perinuclear spaces containing blebs as well as long tube-like evaginations with a bleb at the base, were observed.

*Middle period at 39–40 h after hCG and late period at 50–56 h after hCG.* The NE resembled the NE known from classic descriptions in animal and plant cells with a more regular outline of the two membranes of which it is constituted. Local enlargements of the perinuclear space containing blebs were absent.

In the blastomere nuclei, IAL were always observed. They are frequently in direct continuity with quadruple NE segments (Fig. 9). Blebs are sometimes present within the lumen of the IAL, in the early period (Fig. 10).

The nucleus of the second polar body usually varied during the second cell cycle of the embryo. The PB II nuclei were similar to pronuclei, though much smaller, while in other specimens pycnotic events were already initiated. In PBs lacking signs of pycnosis a few blebs were occasionally found.

In the blastomere cytoplasm small, rough ER vesicles made their appearance while the NE still remained free of ribosomes.

*Third cell cycle*

Approximately 50–56 h after hCG at the beginning of the third cell cycle blebs occurred extremely rarely within the NE of the blastomere nuclei or within the IAL. In the cytoplasm rough ER vesicles were more numerous than in the previous cycle. In the surviving PB II the presence of blebs, also rare, entirely depended on the condition of its nucleus. If the nucleus shows few pycnotic features an occasional bleb, sometimes of giant size, may be present (Figs 11, 12).

*Fourth cell cycle*

In the fourth cell cycle blebs have not been observed either in the nuclei of blastomeres or of the PB II. IAL were more frequent in this cell cycle than in the earlier cycles. Their continuity with the inner leaflet of the NE is sometimes clearly demonstrable (Fig. 13). Ribosomes were attached to the outer leaflet of the NE. Cytoplasmic rough ER vesicles were frequent.

In summary, in fertilized mouse eggs as soon as the nuclear envelope is formed around both pronuclei blebs from the inner leaflet of the NE are formed and project into the perinuclear space. Bleb formation is initiated about 6 h after sperm penetration. Initially, blebbing is frequent but at the middle period of the first cell cycle the number of blebs/10 µm NE length is reduced and blebs disappear entirely prior to mitosis. Blebs are formed again and at the same rate after the blastomere nuclei have been formed in both sister cells. At the middle of the second cell cycle bleb formation ceases, appearing thereafter only sporadically at the third cell cycle, and never later. IAL are formed as a rule at the beginning of the second cell cycle and become more frequent in later cycles. In the second and very early in the third cycle blebs are present within IAL but never seem to leave this space.

*Hybrids*

*Oocyte-thymocyte hybrids.* In cell hybrids between oocytes and thymocytes that are activated a short time after cell fusion (group I), the thymocyte nucleus(i) 'Blebbing' of the nuclear envelope 261
Fig. 8. Large vesicles (arrowhead) form within the nucleus during the late period of the first cell cycle and attach to the inner component of the NE. Within these vesicles bleb-like structures form but are, however, of lower electron density. \( \times 32,000 \). Bar, 0.5 \( \mu \text{m} \).

Fig. 9. In blastomere nuclei during the middle period of the two-cell cycle IAL form (IAL). A flattened vesicle is apposed to the existing NE, forming 'quadruple membranes'. Pores are always absent from these regions. As the inner vesicle turns towards the interior of the nucleus, nuclear pores (arrows) develop on the intranuclear double-membrane portion. \( \times 62,800 \). Bar, 0.25 \( \mu \text{m} \).

Fig. 10. Blebs are formed within the lumen of the IAL. Two-cell stage; middle period. \( \times 62,800 \). Bar, 0.25 \( \mu \text{m} \).

Figs 11-12. At 56 h post-hCG, four-cell stage. Polar body II nucleus shows pycnotic signs but the cytoplasm appears to be healthy. Some giant blebs are observed. Fig. 12 is a higher magnification view of the area outlined in black in Fig. 11. Fig. 11, \( \times 12,500 \); Fig. 12, \( \times 50,000 \). Bar, 0.5 \( \mu \text{m} \).

Fig. 13. At 66 h post-hCG (fourth cell cycle), seven-cell embryo. Frequency of IAL increases and they become a common feature of blastomere nuclei. The IAL are clearly in continuity with the inner leaflet of the NE. Ribosomes are present on NE. \( \times 40,000 \). Bar, 0.5 \( \mu \text{m} \).

Initially lost its NE under the influence of cytoplasmic factor(s) operating in metaphase–anaphase cytoplasm (data not shown, see Szöllősi et al. 1986). The NE was reconstituted around all nuclei at the beginning of the first cell cycle, about 4 h after oocyte activation. At 6 h after activation the female pronucleus and the thymocyte nucleus had fully developed NEs. In every examined hybrid, blebs were observed in both types of nuclei. At 17 h after activation none of the nuclei of the hybrid cell exhibited blebbing.

When the hybrid cell was formed 5–6 h after oocyte activation (group II), the thymocyte nucleus preserved its own NE (Szöllősi et al., unpublished data). Ribosomes were no longer associated with the thymocyte NE, which was the case in the original thymocyte nucleus. In hybrids cultured for 1 h after hybridization the blebbing process was visible in the pronucleus but not in the thymocyte NE. In contrast, after 2–2.5 h of culture following hybridization blebs are rarely present in the donor nuclei (Fig. 14), and sometimes their content is electron-lucent.

Oocyte–blastomere hybrids. In hybrids formed 1 h after oocyte activation or later and cultured for a further 1–6 h (group III), the NE of the blastomere nucleus was not broken down, but the ribosomes lost their association with the NE. The blastomere nucleus could easily be distinguished from the pronucleus because of its irregular shape and its bipartial nucleolus(i) composed of fibrillar
and granular parts typical of nucleoli of multi-cell mouse embryos from the four-cell stage on (Calarco & Brown, 1969). Blebbing started in the pronuclear NE as soon as it was fully formed, i.e. about 4 h after activation with ethanol, as in oocyte–thymocyte hybrids. The blastomere nucleus that was fused with oocytes activated 2 h earlier, and cultured subsequently for 1-5 h, did not show blebbing activity; the pronuclear NE was in the process of formation at that time. When cell hybrids were cultured for longer than 2-5 h, that is when the pronucleus started blebbing, the NE of the blastomere nucleus also showed blebbing activity (Fig. 15). In the proximity of the blastomere nucleus and also at some distance from it, vesicles containing bleb-like structures (Fig. 16) comparable with those already described in the proximity of pronuclei (Fig. 7) are present.

IAL, which are relatively frequent in the blastomere nuclei, remained intact in oocyte–blastomere hybrids also. Blebs were often found in the IAL.

In summary, foreign nuclei started forming NE blebs in hybrid cytoplasm only after the initiation of blebbing in the pronucleus took place; they must stay some time in the host cytoplasm to be 'primed' by the cytoplasm before initiating their own blebbing.

Puromycin treatment

Oocytes in metaphase I cultured with puromycin completed their first meiotic division forming polar body I (PB I) with the nucleus and interphase nucleus in the oocyte. Similar results were obtained by puromycin treatment, as reported by Clarke & Masui (1983). Electron microscopy showed both nuclei with big compact nucleoli. The nuclear envelopes of these nuclei had very few nuclear pores and many cytoplasmic vesicles were in the proximity of the outer leaflet of the NE (Fig. 17). In PB I, extremely numerous perinuclear vesicles were sometimes present (Fig. 18). Neither oocyte nor PB I nuclear envelope forms blebs. No ribosomes were attached to either of the NEs.

In puromycin-treated ovulated oocytes (group 1) many oocytes completed the second meiotic division and two interphase nuclei were formed. In some cases, PB II extrusion was completed, in others two nuclei remained in the oocyte. Nuclei often had irregular, folded outlines. Sometimes vesicles were attached to the NE. The perinuclear space was more or less regular. Some blebs are always present, while some may be of giant size (Fig. 19). Nuclear pores were not frequent. The NE of ovulated oocytes fertilized and cultured in the presence of puromycin (group 2) were similar to pronuclear and PB II nuclear envelopes of oocytes parthenogenetically activated by puromycin (group 1).

Control experiments (groups 3 and 4) examining the effects of puromycin on protein synthesis showed that
methionine incorporation is lower with than without drug (Figs 20, 21).

In summary, nuclei always form blebs after metaphase II activation, but nuclei reconstituted from metaphase I never do. Cytochemical methods using phosphotungstic acid in absolute ethanol did not exhibit PTA binding to any bleb-shaped or bleb-sized structures. This suggests that blebs do not contain basic proteins.

Discussion

This study demonstrates the stage-specific bleb formation along the nuclear envelopes of both pronuclei in the mouse zygote and nuclei during the first half of the two-cell stage. Blebbing does not take place either in the nucleus (germinal vesicle) of mouse ovarian oocytes or in experimentally formed interphase nuclei between meta-
phase I and metaphase II of meiotic maturation. We consider this blebbing to be nucleocytoplasmic transport occurring during limited and specific stages of early mammalian development. As evidence that blebs are a means of transport for some material from nucleus to the cytoplasm, we consider the following: quantitative changes in the number of blebs during the various stages of the first cell cycle, the disappearance of blebs in the middle of the second cycle, and the presence in the cytoplasm at the peak of blebbing activity of structures resembling blebs. The presence of blebs in IAL, which are invaginations of the inner leaflet of the NE (Kessel, 1983), suggests an important role for the inner nuclear membrane. We have never seen blebs in cytoplasmic annulate lamellae (ALs). ALs are derived by either evaginations or 'localized blebbing of the outer nuclear membrane' producing small vesicles first and fusing to form AL later (Kessel et al. 1986). Continuity between the outer membrane of the NE and cytoplasmic stacks of AL is frequently found in sheep and human zygotes (D. Szöllösi, unpublished). All these data indicate that blebbing may represent a particular type of nucleocytoplasmic communication that cannot occur by the conventional route via the nuclear pores. A transport role for blebs has been suggested by other authors (Szöllösi, 1965; Baker & Franchi, 1969; Longo & Anderson, 1969).

Fertilization events (Maro et al. 1984) and synthetic activities during the first two cell cycles have been described in detail in the mouse, under in vitro conditions (Howlett & Bolton, 1985; Howlett, 1986). In our in vitro fertilized oocytes the timing of events is indicated in hours after hCG injection but expressed according to the time schedule established in vitro. Thus we estimate that blebbing of the NE begins and is well under way before DNA synthesis is initiated in the first cell cycle (18–20 h after hCG injection, i.e. 6–8 hpi). In the second cycle blebbing starts at the beginning of G1 as soon as nuclei are formed, while DNA synthesis starts 1–1.5 h later (Howlett, 1986). Thus, bleb formation does not seem to be related to a particular phase of the cell cycle. There is evidence that RNA synthesis or transport are not directly involved in bleb formation. When incorporation of tritiated nucleotides was assessed by electron-microscopic autoradiography, the silver grains were not localized on the pronuclear envelopes and forming blebs (Kopečný, personal communication). Our preliminary analyses using colloidal gold–RNAase complexes also indicate that the blebs do not contain RNA.

Development of blebs in puromycin-treated early rabbit zygote pronuclei has been shown by Longo (1978), and in his experiments protein synthesis was effectively blocked by puromycin. Clarke & Masui (1983) also reported inhibition of protein synthesis in mouse oocytes by puromycin treatment. In our experiments the major result of blocking protein synthesis concerns the difference in blebbing between the nuclei resulting from metaphase I and II spindles under the influence of puromycin. The difference indicates that blebbing is independent of the action of puromycin, whatever it is. It supports the hypothesis that blebbing is typical of the initiation of mammalian development, but that oocyte activation must have taken place, either by the spermatozoon, as happens in vivo, or following the administration of various parthenogenetic agents. The bleb contents do not react with phosphotungstic acid, which causes a specific cytochemical reaction (Courtens & Loir, 1975), suggesting that blebs do not contain basic protein. We are endeavoring in our laboratory to determine what kind of synthetic activity may be involved in bleb formation, when it takes place and what the bleb's fate may be.

Experiments by cell hybridization have shown that blebbing can be induced in donor nuclei, which would never occur in their present differentiated states. After their introduction into the oocyte cytoplasm, however, donor nuclei are induced to do so, when the proper time is chosen. Donor nuclei do not initiate blebbing until the pronucleus starts the blebbing process itself, when they are hybridized close to the time of activation. This suggests that oocyte activation, by either the spermatozoon or ethanol, leads in the end to bleb formation in the NE. Studies on cell hybrids also provided evidence that blebbing may be the consequence and the expression of a dialogue between the nucleus and the cytoplasm in early embryonic development.

The origin of the nuclear envelope is probably not important in determining the capacity of the nucleus to produce blebs. They are produced in nuclei with completely new NE after the initial removal from the thymocyte nucleus. Blebbing occurs also when the NEs of somatic (thymocyte) and embryonic (blastomere) nuclei are retained. In these last cases, the only appreciable difference between the NE of donor nuclei before and after fusion is the loss of polysomes from the NE surface under the influence of the ooplasm. The 2 h period of minimal exposure of donor nuclei to the activated ooplasm that is required before the initiation of blebbing must be connected to some processes other than NE exchange.

In both in vitro fertilized oocytes and parthenogenetically activated cell hybrids blebbing activity has a limited duration. A slowing down in the blebbing rate in the first cell cycle starts as cleavage approaches and blebbing is almost absent by about 28–34 h post-hCG (16–22 hpi), i.e. just before NE breakdown. A similar schedule was observed in oocyte–thymocyte hybrids. In the second cell cycle blebs were formed only during the first few hours following cleavage, i.e. during the initiation of genome activity. The earliest but limited activation of the embryonic genome takes place at the beginning of the second cell cycle and the major activation is between 26 and 29 hpi in the mouse (Flach et al. 1982), i.e. between 6 and 9 h of the second cell cycle.
The mechanism for controlling bleb formation has not been investigated. Our results clearly indicate that bleb formation in the mouse characterizes the first and second cycles but not the phase of the cycle itself. It starts with activation of the oocyte, either by the fertilizing sperm or by the parthenogenetic stimulus, and overlaps the initiation of the embryonic genome expression. Blebbing activity may therefore be an expression of the removal and discarding of controlling elements associated with the chromatin, in order to permit the expression of new genes, instead of being a transfer of information as we suggested above.

Blebs in the NE, which are reminiscent of the blebs described in early mouse embryos have been described sporadically in certain other cells. In oocytes of the ascidian *Botrylloides viridis* (Hsu, 1967), in haemocytoblasts of the 14-day-old rabbit embryo, in cells of the *stratum germinativum* of hamster epidermis, the shoot apex of corn, *Zea mays* (Hadek & Shift, 1962), and in the degenerating gustatory cells from rabbit foliate papillae (Scalzi, 1967) similar blebbing activities have been reported. They may represent other examples of the highly specific nuclear transport system involved in different cellular processes.

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References


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