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BEHAVIOUR OF THYMOCYTE NUCLEI IN NON-ACTIVATED AND ACTIVATED MOUSE OOCYTES

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SUMMARY

Cells originating from the thymus of newborn mice were fused with mouse oocytes using polyethylene glycol. The behaviour of thymocyte nuclei was studied in non-activated metaphase II oocytes, and in oocytes activated *in vitro* with ethanol.

In non-activated oocytes all thymocyte nuclei undergo premature chromosome condensation with individualization of chromosomes; the chromosomes form separate groups in the cytoplasm, or are assembled around the metaphase II spindle, or located on the extra-spindle.

In activated oocytes thymocyte nuclei start to develop along a pronucleus-like pathway (decondensation, visualization of nucleoli, swelling) and increase up to 200 times in volume during 24 h culture *in vitro*, eventually reaching the size of a fully grown pronucleus. Activation/fusion timing seems to be critical for the full remodelling of thymocyte nuclei. Nuclei introduced before (10–30 min) or shortly after (up to 60 min) activation often grow larger than the female pronucleus. Those introduced into oocytes long before activation (>30 min) undergo premature condensation with subsequent reformation of nuclei that are sometimes deficient (as indicated by the presence of micronuclei), or of hybrid character. Nuclei introduced late after activation (>60 min) are mostly doomed to retarded development.

The implications of the present observations for nuclear transfer experiments in mammals are discussed.

INTRODUCTION

Despite the failure to obtain adult animals from nuclear transplants carrying 'adult' nuclei, experiments on amphibians provide evidence that at least some nuclei from differentiated cells can be reprogrammed in the egg cytoplasm and exhibit genetic pluripotency (for a review see DiBerardino, 1980).

In mammals, the developmental potential of embryonic nuclei from preimplantation embryos was tested in the rabbit and in the mouse, by microsurgical transfer of nuclei into artificially activated (Bromhall, 1975) or fertilized eggs (Modliński, 1978, 1981). The genetic totipotency of embryonic nuclei was proved by the experiments of Illmensee & Hoppe (1981*a*,*b*), who succeeded in obtaining adult fertile mice, after transplantation of nuclei from the inner cell mass of a blastocyst and from the embryonic ectoderm of a 7-day mouse embryo into fertilized and enucleated eggs.

So far, the developmental potency of post-embryonic nuclei has not been tested in mammals. However, experiments using virus-mediated cell fusion have demonstrated that 'differentiated' somatic nuclei can undergo partial remodelling (decondensation of chromatin, swelling) in the cytoplasm of activated mouse eggs (Graham, 1969;

Tarkowski & Balakier, 1980), and are reprogrammed under the influence of cytoplasmic factors present in two- and four-cell stage blastomeres (Bernstein & Mukherjee, 1973). The decisive role of the cytoplasm in controlling the behaviour of nuclei has been shown in mammals in cell fusion experiments on oocytes, eggs and blastomeres (for a review see Tarkowski, 1982).

Before undertaking experiments aimed at testing the potency of post-embryonic nuclei to promote development in mammals, we considered it essential first to carry out cytological studies on the behaviour of such nuclei in the egg cytoplasm during the first cell cycle. In the present study we demonstrate that thymocyte nuclei introduced into the cytoplasm of artificially activated mouse eggs can simulate the pattern of changes characteristic of the egg's own pronuclei during normal development, and that activation/fusion timing appears to be critical for their morphological remodelling.

MATERIALS AND METHODS

Oocytes (recipients)

Oocytes were obtained from F1 (C57Bl/10×CBA-H), A, 129/ter Sv, C57Bl/10 and CBA-T6T6 2 to 12-month-old female mice that were ovulating spontaneously (mating with vasectomized males, dissection between 8.30 and 10.00 a.m.), or induced to ovulate (5–10 i.u. of each of pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG), given 46–56 h apart, dissection 13½ to 16½ h post-hCG). After dispersing the cumulus cells with hyaluronidase (200–300 i.u. per ml), zona-free oocytes (zona digested with 0·5 % Pronase) were placed in drops of Whitten's (1971) medium under liquid paraffin, and cultured for 15 min to 3 h (37 °C, 5 % CO_2 in air) before further manipulation.

Activation

Ovulated oocytes were activated 15–20 h post-hCG with 7–8 % ethanol (prepared from 96 % ethanol for spectroscopy) in Whitten's medium for 5 min at room temperature, under liquid paraffin, according to the method of Cuthbertson (Cuthbertson, Whittingham & Cobbold, 1981; Cuthbertson, 1983) inspired by observations of Dyban & Khozhai (1980). After thorough washing in Whitten's medium, oocytes were cultured in separate drops of culture medium according to the procedure given above, and inspected during the next 2–3 h for signs of activation manifested by the extrusion of the second polar body, or the occurrence of 'immediate cleavage' or fragmentation. The activation rate in the majority of experiments was higher than 80 %.

Thymocytes (donors)

Thymus of 1 to 7-day-old mice was used as a source of cells. In newborn mice the thymus is largely populated with primitive (young) forms of lymphocytes (thymocytes), rapidly proliferating with a cell division rate of 6–8 h (Rygaard, 1973). Thymocytes measure approx. 7–11 μm in diameter and carry a large nucleus surrounded by a thin rim of cytoplasm. The nucleus contains one to two nucleoli, which become rudimentary as the cells transform into mature lymphocytes. Since the lymphocytes account for up to 90 % of the weight of the thymus, we believe that the cells used for fusion with oocytes were predominantly of lymphocyte origin.

Bi-lobed thymus was carefully cleaned of adhering tissues and coagulated blood, washed several times in phosphate-buffered saline (PBS), and stored in bovine serum albumin (BSA)-free medium (i.e. medium 16; Whittingham, 1971) buffered with HEPES (Fulton & Whittingham, 1978), in agar-coated embryological watch-glasses (1 % agar in 0·9 % NaCl), at room temperature. Cutting of thymic lobes into tiny pieces resulted in rapid dissociation of spongy thymic tissue and accumulation of liberated thymocytes on the bottom of the dish (Figs 1, 2).

Cell manipulation and cell fusion

All manipulations (except treatment with polyethyleneglycol (PEG)) were performed in agar-coated embryological watch-glasses, at room temperature. Non-activated and activated zona-free oocytes were thoroughly washed in BSA-free M2 medium, and treated for 2–10 min with phytohemagglutinin (PHA; Sigma, 100–150 μg/ml in BSA-free M2 medium). The PHA-treated oocytes were then placed on, and rolled over the monolayer of thymocytes (Fig. 1) until it was evident that numerous cells were firmly adhered to their surface. These aggregates were treated again with PHA for several minutes and immersed in freshly prepared 50 % PEG (Loba-Chemie, M_r 1000) in twice-concentrated BSA-free M2 medium (w/v) for 45–60 s.

In addition, in a few pilot experiments single thymocytes were microsurgically transferred into the perivitelline space of intact pronucleate eggs, both fertilized and artificially activated (Figs 3, 4). The oocytes that had been operated on were either incubated in PHA (100–150 μg/ml) or the lectin was injected into the perivitelline space, and then they were treated with 45 % or 50 % PEG (Loba-Chemie, M_r 1500) in twice-concentrated BSA-free M2 (w/v) for 45–75 s.

Following treatment with PEG, zona-free and zona-surrounded oocytes were carefully washed in BSA-free M2 and in Whitten's medium. They were cultured according to the standard procedure for 30 min to 25 h, and subsequently processed for haematoxylin-stained whole mounts (Tarkowski & Wróblewska, 1967).

The diameter of the nuclei was measured on whole-mount preparations, with a 15× eyepiece micrometer, under a 40× objective.

RESULTS

Following treatment with PEG, fusion of cell membranes occurs, leading to the introduction of thymocyte nuclei (th-nuclei) and cytoplasm into recipient oocytes. The number of nuclei introduced in this way varied from 1 to 15 per oocyte, with numerous unfused thymocytes attached to the oocyte cell membrane.

The behaviour of th-nuclei was studied in: (1) non-activated oocytes subjected exclusively to fusogenic PEG treatment; (2) activated oocytes: (a) first subjected to the fusion procedure, and then activated; (b) first activated, and then subjected to the fusion procedure.

Thymocyte nuclei in non-activated oocytes

All thymocyte nuclei introduced into the cytoplasm of non-activated metaphase II oocytes underwent premature condensation. One to 15 condensed th-nuclei could be identified in the cytoplasm, although their number might have been underestimated when large aggregates of condensing thymocyte chromosomes (th-chromosomes) were present (Fig. 5). As early as 30 min after PEG treatment, the first nuclei showed the initial stages of chromatin condensation (Fig. 6). After 45 min the full spectrum of the condensation process was observed, and about 1 h after PEG treatment the majority of nuclei had reached the stage of prematurely condensed chromosomes (PCC) in metaphase configuration (Fig. 7). In heterokaryons cultured for a few hours, PCC tended to shorten (rod-like appearance) and to disperse in the cytoplasm.

Prematurely condensed chromosomes were found at the periphery or inside oocytes. Occasionally, they were grouped around the metaphase II spindle (6 out of 46 oocytes), or located on the extra-spindle of bipolar or multipolar organization (7 out of 46 oocytes) (Figs 8, 9). Such variable behaviour of prematurely condensed

chromosomes has been described previously, in both mammalian (Tarkowski & Bałakier, 1980; Szöllösi, Bałakier, Czołowska & Tarkowski, 1980) and amphibian oocytes (Gurdon, 1968; Leonard, Hoffner & DiBerardino, 1982).

Thymocyte nuclei in activated oocytes

The majority of oocytes subjected to ethanol shock reacted by extruding the second polar body in about 2 h after treatment and forming a single haploid pronucleus. The remaining activated oocytes reacted abnormally by undergoing 'immediate cleavage' or fragmentation, or by suppressing the second polar body with the formation of two haploid nuclei. It appears that when ethanol and a fusogen are applied with a short time interval, activation can be delayed and the incidence of atypical reactions increases. In the majority of haploid eggs with a second polar body, the female pronucleus contained a single, large, centrally situated nucleolus and this organization was retained during the first day of culture *in vitro*.

Fusion was often indicated by the formation of a protrusion resembling a 'fertilization cone' (Fig. 10), which probably reflects changes in the cortical layer associated with the incorporation of the foreign cell membrane and/or cell cytoplasm into the recipient egg.

Th-nuclei introduced into oocytes at the time of activation maintained their interphase character. The first reaction detected was the rounding up and decreased stainability of the nucleus. Subsequently, patches of deeply stained material appeared underneath the nuclear membrane followed by the individualization of nucleolus-like

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Abbreviations and symbols used in figures: *th*, thymocyte; *MII*, oocyte chromosomes in metaphase II; *pb*, second polar body; ♀, female pronucleus; p.a., post-activation.

Fig. 1. Non-activated zona-free oocytes (cell size, 75 μm) placed over the monolayer of thymocytes. ×148.

Fig. 2. Monolayer of thymocytes obtained from newborn mice (cell size, 7–11 μm). ×400.

Figs 3, 4. Microsurgical transfer of a thymocyte into the perivitelline space of an intact egg. The egg is immobilized with a holding pipette (left) and the thymocyte is inserted into the perivitelline space with an injection pipette (right). ×250.

Fig. 3. Penetration through the *zona pellucida*. The thymocyte is still inside the pipette.

Fig. 4. The injected thymocyte adheres to the PHA-treated egg surface close to the puncture in the *zona pellucida*.

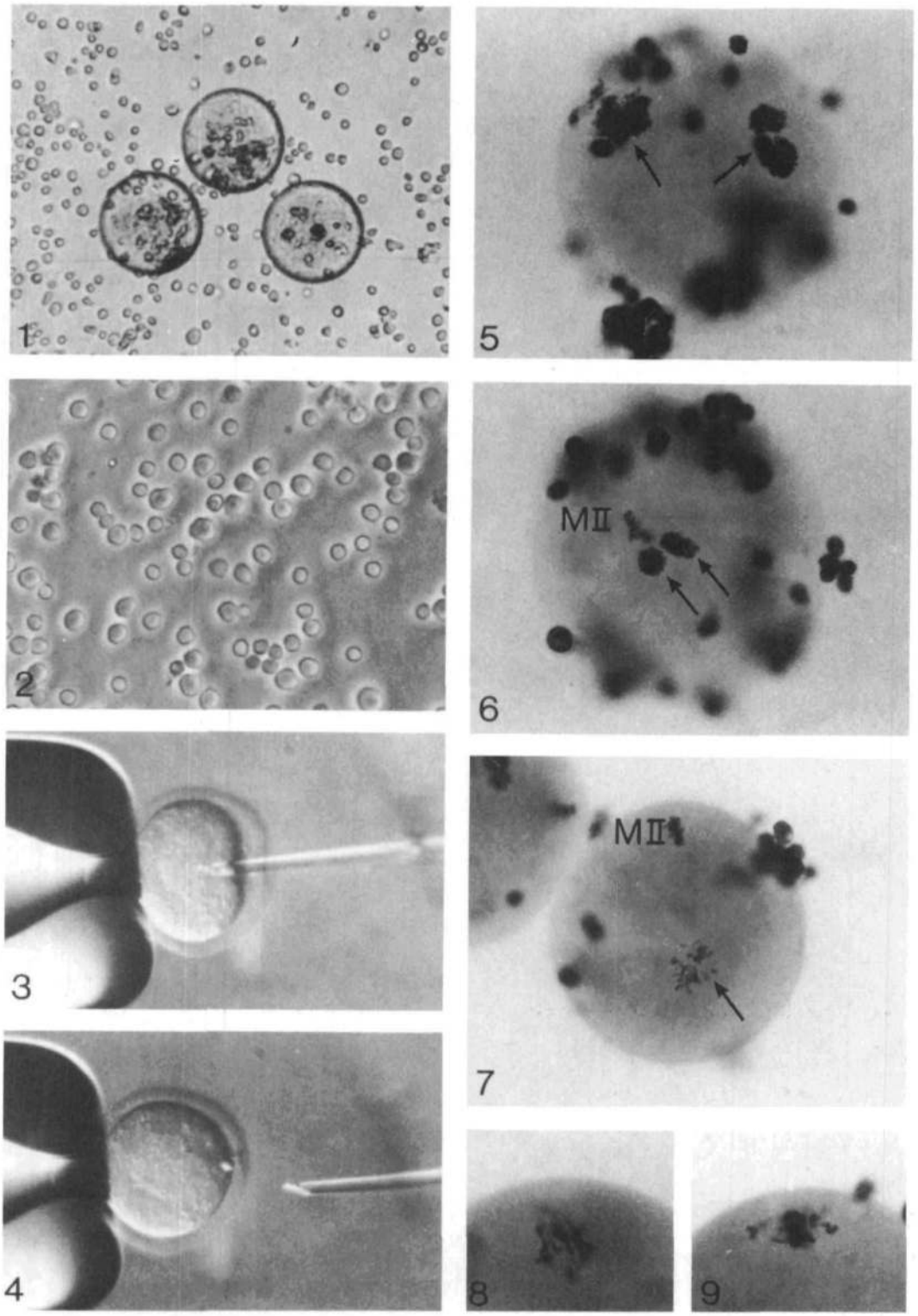
Figs 5–9. Premature chromatin condensation (arrows) in interphase th-nuclei introduced into non-activated metaphase II oocytes. ×1250.

Fig. 5. Large aggregates of condensing th-chromosomes. Meiotic metaphase II masked by th-chromosomes (1 h culture).

Fig. 6. Early post-fusion condensation changes in th-nuclei situated in the vicinity of meiotic metaphase II (30 min culture).

Fig. 7. th-chromosomes in metaphase configuration (centrally situated). Near the surface are oocyte chromosomes in metaphase II (1½ h culture).

Figs 8, 9. Thymocyte chromosomes aligned on an extra-spindle, which was induced in a metaphase II oocyte following fusion with thymocytes. Prematurely condensed chromosomes are scattered along the spindle and at the poles (1 h and 2½ h culture, respectively). ×1900.



Figs 1–9

structures (Fig. 11A–J). Nucleoli increased in number with time, up to six to eight in fully remodelled (i.e. displaying a pronucleus-like appearance) th-nuclei. These changes were accompanied by chromation decondensation and nuclear swelling (Fig. 11A–J), with the th-nucleus chromatin staining more intensely, as a rule, than that of the female pronucleus. The remodelled th-nuclei progressively attained the size of a grown female pronucleus, thus increasing in volume up to 200-fold after 20–24 h culture. In parthenogenetic eggs approaching the first cleavage division, remodelled th-nuclei frequently resembled late pronuclei so closely (decreased number of nucleoli, thread-like organization of chromatin) that the two types of nuclei became indistinguishable from each other. We do not know whether such pronucleus-like th-nuclei can participate in the mitosis of the first cell cycle, because in our experimental system the eggs were checked at the late pronucleate stage of development.

In order to define the optimal conditions ensuring full remodelling of th-nuclei, heterokaryons were produced in different experimental variants by manipulation of the activation/fusion sequence and the time-interval between these two treatments (see Table 1).

Fig. 10. Following fusion with a thymocyte (PEG 60 min p.a.), the activated oocyte developed a large protrusion (arrow) containing two th-nuclei (one highly condensed, one slightly decondensed). Five other condensed th-nuclei are also present in the oocyte. The arrowhead indicates one group of telophase chromosomes. The second polar body (*pb*) and the second group of telophase chromosomes are slightly out of focus (1 h 40 min culture post-PEG). ×1250.

Fig. 11A–J. A series of thymocyte nuclei in activated oocytes showing sequential morphological changes characteristic of the remodelling and formation of a pronucleus-like nucleus (nuclei originate from different oocytes). Remodelling comprises appearance of nucleolus-like structures, chromatin decondensation and nuclear swelling. ×2000.

Figs 12–16. Development of thymocyte nuclei (*th*) introduced into metaphase II oocytes before artificial activation. ×1250.

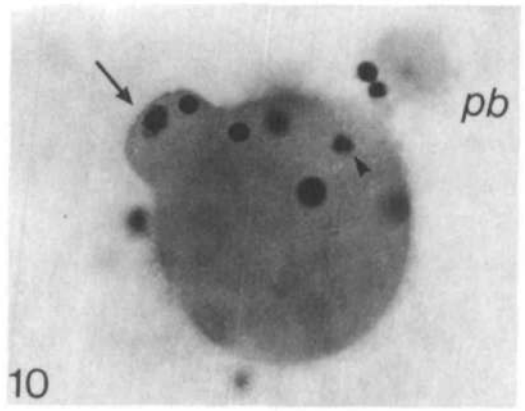
Fig. 12. In an oocyte activated 15 min after PEG treatment, the th-nucleus grew larger than the female pronucleus; a small micronucleus present in the oocyte is not shown. The figure is mounted from two photographs taken at different focal planes (19 h culture p.a.).

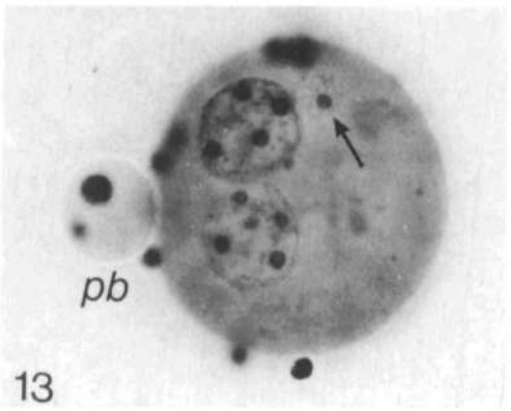
Fig. 13. An oocyte activated 15 min after PEG treatment contains two large multinucleolar nuclei of similar size and structure. A third smaller nucleus (arrow) may represent a retarded female pronucleus, or a micronucleus. Another interpretation is that the oocyte chromosomes became incorporated into one or two nuclei together with th-chromosomes (differentiated after rapid cell fusion) thus forming a hybrid nucleus(i) (19 h culture p.a.).

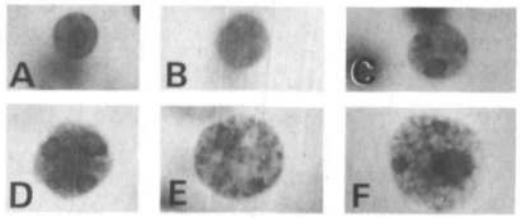
Fig. 14. An oocyte activated 25 min after PEG treatment. Minute micronuclei (arrows) beside the swollen th-nucleus probably originated from the prematurely condensed th-chromosomes that were not incorporated into the reforming nucleus. The female pronucleus is smaller than the th-nucleus (25 h culture p.a.).

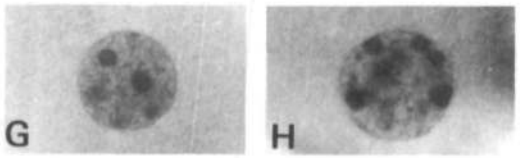
Fig. 15. Fragment of an oocyte subjected to delayed activation (100 min) after PEG treatment, showing two nuclei of different size and morphology: the egg pronucleus (♀), and the th-nucleus *th*. Note the presence of an extra-polar body (*epb*), which was probably formed as a result of mitosis of th-chromosomes arranged on an extra-spindle. Uneven cytokinesis of the meiotic-division type is due to subcortical localization of thymocyte spindle (cf. Figs 8, 9) (8 h culture p.a.).

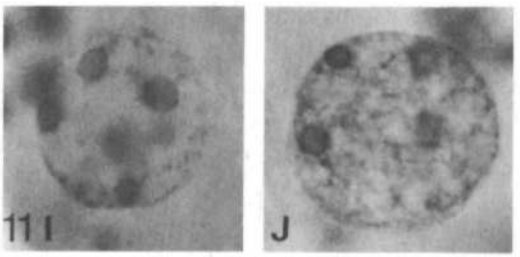
Fig. 16. Single large multinucleolar nucleus, probably represents a hybrid nucleus reconstituted from oocyte and th-chromosomes following activation. Second polar body slightly out of focus (activation 15 min post-PEG, 19½ h culture p.a.).

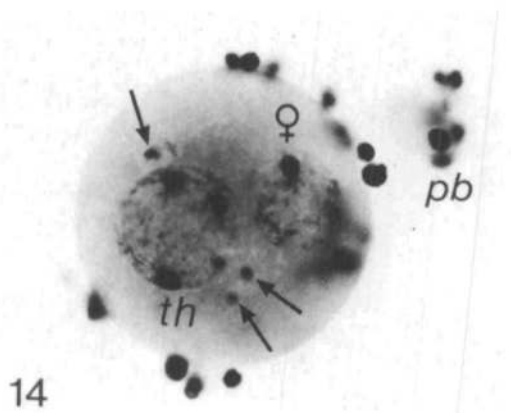


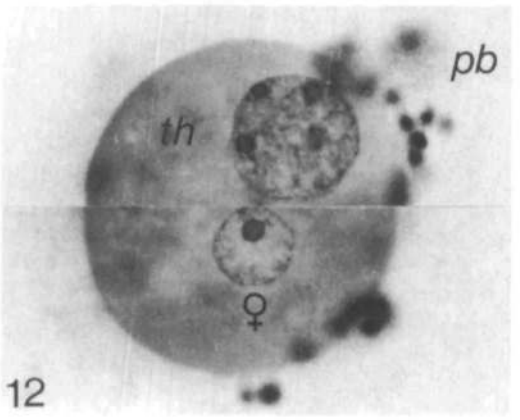


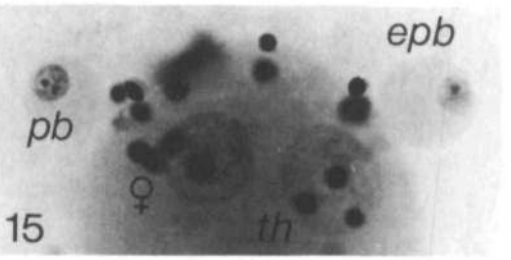


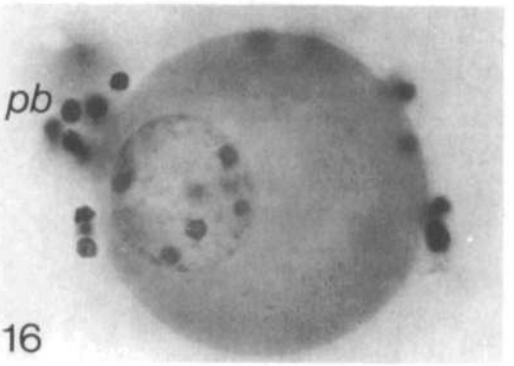












Figs 10–16

Table 1. *Behaviour of thymocyte nuclei introduced into non-activated and activated oocytes*

Experimental group		Total no. of heterokaryons	No. of groups of condensing chromosomes	Size relations between th-nuclei (th-n) and female pronucleus (f-n) in individual oocytes* (No. of heterokaryons (%))					No. of heterokaryons with hybrid nuclei†
Oocytes	Treatment			th-n > f-n	th-n ‖ f-n	f-n > th-n	Total	th-n = f-n	
Non-activated	PEG	46	118+70‡						
Activated	>30 min PEG+act	12		4		1	5	2	5
Activated	<30 min PEG+act	22		14		2	16	4	2
Activated	<60 min act+PEG	70+11§		31 (62)	3 (6)	16 (32)	50 (100)	20	
Activated	>60 min act+PEG	33		6 (19)	4 (12)	22 (69)	32 (100)	1	
	Total	183+11§		55	7	41	103	27	7

PEG, fusogen treatment; act, activation; 30 min, etc. is interval time; th-n>f-n, thymocyte nucleus larger than female pronucleus; th-n ‖ f-n, parallel development of nuclei; f-n>th-n, female pronucleus larger than thymocyte pronucleus; th-n=f-n, fully grown, indistinguishable nuclei.

* Nuclear diameter of most advanced nuclei in oocytes and oocyte fragments.

† Classified on the basis of nuclear morphology.

‡ Multinucleate heterokaryons, approximation.

§ Oocytes in telophase.

Nuclei introduced into oocytes before activation. In this variant only a small number (usually 1–2, occasionally up to 5) of th-nuclei were introduced, probably due to the destructive action of ethanol on those cells that had failed to fuse before the activation procedure.

The response of th-nuclei introduced into metaphase II oocytes that were subsequently activated was uniform: all th-nuclei underwent remodelling with the formation of decondensed swollen nuclei.

In Table 1 the behaviour of remodelled thymocyte nuclei (th-n) *versus* the host female pronucleus (f-n) is compared on the basis of their size (diameter) in heterokaryons cultured for 4–25 h. If the interval between PEG treatment and activation did not exceed 30 min, growth of th-nuclei could have been even faster than that of the pronucleus; in the majority of hybrids studied (14/16) one to two swollen large th-nuclei resided beside the developmentally retarded host pronucleus (th-n > f-n) (Figs 12–14). It seems that prolongation of the time between the two treatments (>30 min) increases the frequency of oocytes with abnormally reconstituted nuclei (hybrid nuclei, micronuclei). In one egg an extra polar body situated close to the th-nucleus was observed (Fig. 15). This 'polar body' was probably formed as a result of mitosis of th-chromosomes arranged on an extra-spindle (cf. Figs 8, 9).

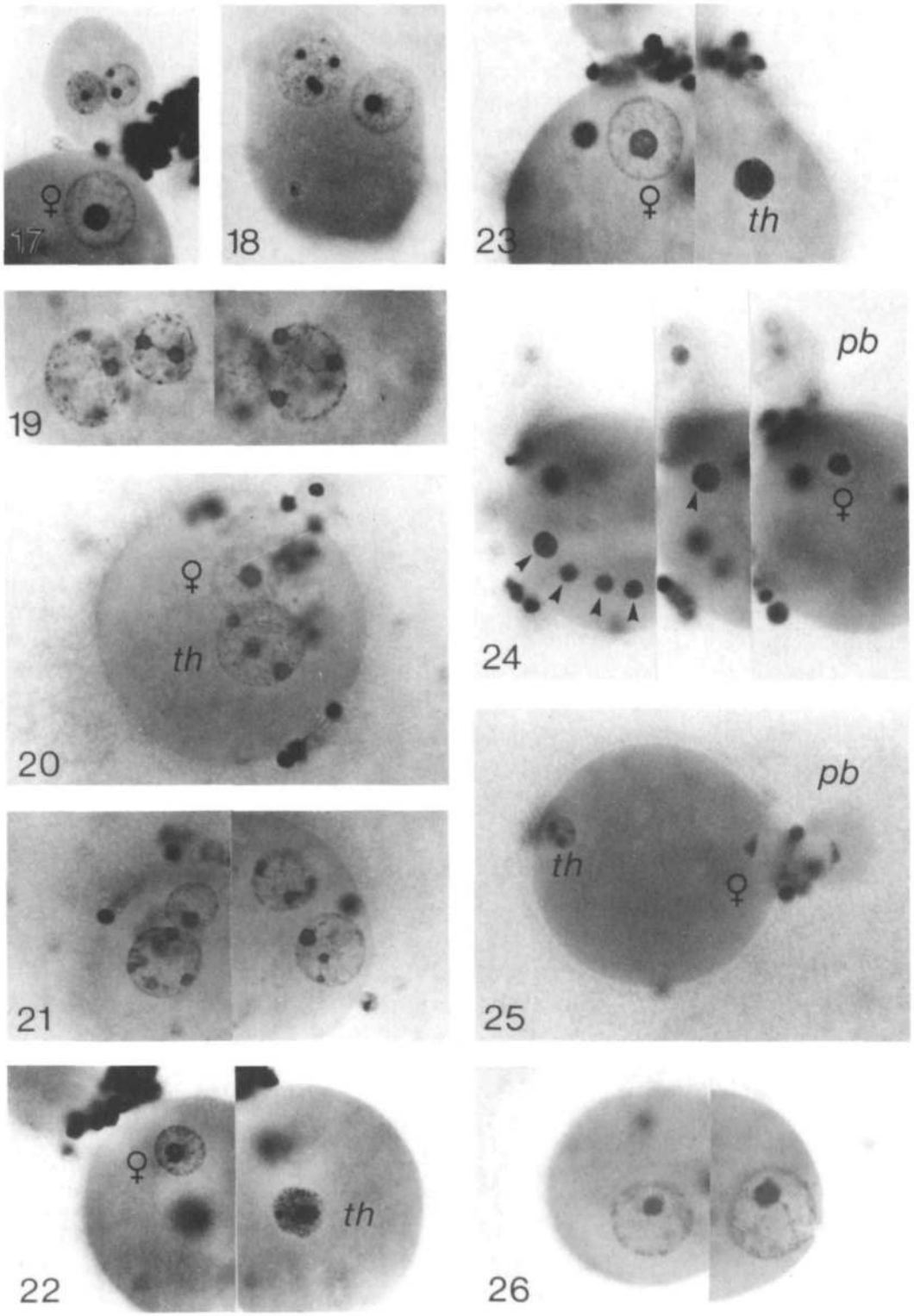
In the oldest heterokaryons, cultured for 24–25 h, it was not always possible to distinguish somatic nuclei from the egg pronucleus by morphological criteria, and these eggs are grouped separately in Table 1 (th-n = f-n).

In several cases, the morphology of the only nucleus in the oocyte or of the nucleus in one of the cells of an 'immediately cleaved' oocyte was of the remodelled th-nucleus type, suggesting that a true hybrid nucleus was reconstituted from 'embryonic' and 'somatic' chromosomes (Fig. 16).

Nuclei introduced into activated oocytes. In this experimental variant a large number of nuclei (up to 12) were introduced into oocytes. th-nuclei have been also found in the second polar body (Fig. 17), in cells resulting from immediate cleavage, and in minicells of fragmented eggs (Fig. 18). The sizes of remodelled nuclei were proportional to the volume of the cell in which they resided. Another characteristic of this experimental variant is that apparently remodelled th-nuclei may co-exist with unchanged nuclei, or nuclei displaying only early post-fusion changes. As in a previous variant, a large portion of hybrids cultured during 22–25 h contained nuclei whose origin could not be defined on the basis of nuclear morphology (th-n = f-n, Table 1) (Fig. 19).

Optimal growth of th-nuclei seems to be determined by the time interval between the application of the activating shock and PEG treatment, with a 1-h delay being the critical demarcation; in fusions induced later th-nuclei are usually poorly remodelled and lag behind the pronucleus. As shown in Table 1, in 62 % of heterokaryons treated with PEG up to 60 min post-activation (p.a.), and cultured for 4–25 h afterwards, the development (swelling) of th-nucleus(i) was faster than that of the egg pronucleus (th-n > f-n) (Figs 20, 21), and in 6 % of the cases parallel development of both types of nuclei was observed (th-n ‖ f-n) (Fig. 22). The remaining heterokaryons had a well-developed female pronucleus and a developmentally retarded th-nucleus(i) (f-n > th-n) (Fig. 23).

The reaction of th-nuclei was different if the treatment with PEG was delayed



Figs 17–26

beyond 60 min after activation (>1; 2, 3 and 6 h). The longer the post-activation time, the smaller the proportion of remodelled th-nuclei; and the majority of them did not progress, during 3–6 h of culture, beyond the initial stages of the post-fusion response (rounding up, decreased stainability) (PEG up to 3 h p.a., remodelled nuclei in 13/27 heterokaryons; PEG 6 h p.a., remodelled nuclei in 1/6 heterokaryons). In these oocytes the f-n > th-n type of development of nuclei predominated (69% of heterokaryons, Table 1) (Fig. 24). Several cases with the th-n > f-n, or th-n ‖ f-n patterns of development were usually found in eggs showing atypical post-activation reaction (see p. 22) and can be explained by delayed oocyte activation.

Figs 17–25. Behaviour of thymocyte nuclei introduced into previously activated oocytes. ×1250.

Fig. 17. In the large second polar body of an oocyte fused with a thymocyte 47 min p.a., a slightly remodelled th-nucleus with denser chromatin (left) resides beside the nucleus of the second polar body. The female pronucleus is located underneath the *pb* (5½ h culture post-PEG treatment).

Fig. 18. Mini-cell of a fragmented oocyte treated in the same way as that shown in Fig. 17, with two nuclei of different morphology. The multinucleolar, denser nucleus (left) is considered to be a remodelled th-nucleus. The second female pronucleus is present in another fragment (not shown).

Fig. 19. An oocyte cultured for 23 h post-fusion (PEG treatment 25 min p.a.) contains three nuclei of similar morphology that make it impossible to distinguish the female pronucleus from the remodelled th-nuclei (mounted picture).

Fig. 20. A large th-nucleus can be distinguished from the female pronucleus (which is slightly out of focus) due to its morphology (th-n > f-n type of development; see text); oocyte treated with PEG 50 min p.a., and cultured afterwards for 24 h.

Fig. 21. Fragment of an oocyte treated similarly to that shown in Fig. 20. Out of four nuclei, the smallest nucleus is classified as the pronucleus, and three larger nuclei as remodelled th-nuclei (th-n > f-n type of development; see text). The difference in the size of these nuclei in comparison to those shown in Fig. 20 may be due to the difference in the total numbers of nuclei in these two oocytes. The figure is mounted from two photographs taken at different focal planes.

Fig. 22. Parallel type of development of a pronucleus and a *th*-nucleus (th-n ‖ f-n) observed in an activated oocyte subjected to PEG treatment 48 min p.a. (mounted picture). The nucleus of thymocyte origin (right) is characterized by dense chromatin (6½ h culture post-PEG treatment).

Fig. 23. The female pronucleus dominates the th-nucleus (f-n > th-n type of development). The large swollen pronucleus is accompanied by a th-nucleus (right) that shows only early remodelling changes. The second th-nucleus present in the same oocyte (out of focus) is even less advanced in the remodelling process (oocyte fused with thymocytes 55 min p.a., and cultured for 5 h).

Fig. 24. Behaviour of th-nuclei introduced into an oocyte 3 h p.a. (mounted picture). Several th-nuclei (arrowheads), which have entered the oocyte, display only initial stages of post-fusion response (rounding up, decreased stainability), whereas the female pronucleus is decondensed and slightly swollen (3 h culture post-PEG treatment).

Fig. 25. An oocyte in telophase of the second maturation division showing remodelled th-nucleus (nucleoli visible, chromatin decondensation) (fusion 33 min p.a., 3 h culture after PEG treatment).

Fig. 26. An egg first activated, then microsurgically injected with a single thymocyte and subjected to PEG treatment (PEG, 1 h 40 min p.a.). The th-nucleus and female pronucleus are indistinguishable (mounted picture). The second polar body has disintegrated during culture, but its presence was ascertained during *in vitro* examination under the Nomarski microscope (23 h culture after PEG treatment). ×1250.

Behaviour of th-nuclei during completion of the second meiotic division

The oocytes that fused with the thymocytes before the extrusion of the second polar body (PEG 30–60 min p.a.), were studied 2–3 h later, i.e. at the time of completion of the second meiotic division. In oocytes in anaphase/telophase th-nuclei retained, as a rule, their thymocyte morphology with condensed chromatin, or underwent the first visible change (decreased stainability of chromatin, visualization of nucleoli, occasionally minimal swelling) (Fig. 25). In eggs with a minute, just reconstituted pronucleus (nuclear membrane and nucleolus visible), apparently decondensed th-nuclei were detected. The significance of this finding will be considered later in the Discussion.

Thymocytes injected under the zona pellucida

In pilot experiments with microsurgical transfer of single thymocytes under the *zona pellucida* (see Materials and Methods, p. 21) the frequency of fusion was low. Of the 134 oocytes that survived both the operation and PEG treatment, 13 oocytes contained a thymocyte nucleus. Depending on the time that elapsed between oocyte activation and cell fusion, th-nuclei either underwent full remodelling (Fig. 26) or were developmentally retarded.

DISCUSSION

Fusion experiments using somatic and embryonic cells (for reviews see, respectively, Ringertz & Savage, 1976; Tarkowski, 1982) have shown that nuclear behaviour and nuclear activity are determined by the state of the cytoplasm in the partners undergoing fusion. However, the fusion experiments described in this paper are more comparable to microsurgically achieved nuclear transfer, because the amount of thymocyte cytoplasm introduced into the oocytes is negligible.

Since the exact time of the fusion of thymocytes with oocytes cannot be defined precisely, timing in our experiments is expressed in relation to PEG treatment. However, it can be estimated roughly that the first thymocyte nuclei enter the oocyte cytoplasm about 15–30 min after PEG treatment, and that majority of fusions occur within the first hour.

The behaviour of thymocyte nuclei in oocytes is evidently influenced by the developmental state of the oocyte. In non-activated oocytes in metaphase II, th-nuclei undergo premature chromosome condensation. This finding confirms earlier results obtained with amphibians (DiBerardino, 1980) and mammals (Tarkowski & Bałakier, 1980). Premature chromosome condensation is induced by a cytoplasmic factor present in meiotic oocytes, which seems to be a universal factor operating in both meiotic and mitotic cells (mammals, Bałakier, 1978; for reviews see Masui & Clarke, 1979; DiBerardino, 1980; Rao, 1982).

Activation releases the oocyte from the metaphase block and permits reconstitution of interphase nuclei. Depending on the organization of prematurely condensed

chromosomes (PCC) in non-activated oocytes, their further post-activation fate may follow one of four pathways:

(1) Integrated chromosome group → reformation of a complete (diploid) nucleus;
(2) Dispersed chromosome group → aneuploid nucleus + micronuclei;
(3) PCC associated with the meiotic spindle → formation of a hybrid nucleus composed of 'embryonic' and 'somatic' chromosomes;
(4) PCC on the extra-spindle → reformation of a diploid or an aneuploid nucleus; formation of an extra-polar body?

It seems that only pathway (1) (and possibly (4)) may ensure reformation of a diploid somatic nucleus. However, if the time interval between fusion and activation is very short, the th-nucleus probably escapes the chromosome-condensation stage and enters directly into the phase of remodelling. There are indications that such a nucleus has a developmental advantage over a female pronucleus (see below).

Whereas all th-nuclei introduced into non-activated oocytes undergo morphological remodelling following release from the metaphase II block (and irrespective of whether or not they pass through chromosome condensation), those introduced into previously activated oocytes behave in a diverse manner. As a rule they retain interphase organization, but their further fate depends on time relations between activation and fusion. The shorter the interval between these two events, the higher the chance that the thymocyte nucleus will initiate development concomitantly with or even before that of the pronucleus and, consequently, that it will undergo full remodelling (i.e. follow the pattern of changes characteristic of a pronucleus) (Table 1). The critical moment up to which remodelling of foreign nuclei is still possible can be estimated to be at about 1 h post-activation. In nuclei introduced later the degree of remodelling declines with time, and an increasing proportion of them show developmental arrest.

Activation generates an abrupt change in the physiological state of the oocyte cytoplasm from condensation-inducing towards interphase-preserving activity. However, the reconstitution of the egg pronucleus cannot start until the second meiotic division is completed. If the foreign nuclei enter the egg cytoplasm during this particular period (activation–extrusion of the second polar body), they retain their interphase character although the meiotic division is still in progress. This interphase status of th-nuclei enables them to decondense and swell before the pronucleus does. If, however, the foreign nuclei are introduced after the pronucleus has been reconstituted and begun to swell, they are doomed to retarded development. These findings are in accord with the observation that in parthenogenetic eggs undergoing delayed 'fertilization' the degree of transformation of the sperm nucleus into a male pronucleus is inversely correlated with the time interval between egg activation and sperm penetration (Komar, 1982; Tarkowski & Tittenbrun, unpublished observations). These two pieces of evidence, together with the results of our earlier studies on the fate of the sperm nucleus in nucleate and anucleate oocyte fragments produced before and after germinal vesicle breakdown (GVBD) (Bałakier & Tarkowski, 1980), permit us to formulate the following explanation of the phenomena observed: (1) the extrachromosomal constituents of the karyoplasm of the germinal vesicle (most probably

nuclear-specific proteins) that enter the oocyte cytoplasm following GVBD are essential for nuclear decondensation and swelling; (2) after egg activation these proteins are gradually absorbed by the decondensing and swelling nuclei until the pool becomes exhausted; (3) any nucleus (th-nucleus, female pronucleus, sperm nucleus, etc.) that starts decondensation and swelling beyond a certain time limit (not yet defined precisely), in relation to the other interphase nucleus already present in the egg, is handicapped in the competition for nuclear proteins and, consequently, develops abortively. It is worth recalling that in the present experiments the degree of swelling of thymocyte nuclei depended both on the activation–fusion interval and on the number of nuclei introduced into the oocyte. This supports the idea that there is competition between the growing nuclei for the uptake of nuclear-specific proteins from the cytoplasmic pool, similar to that observed in polyspermic eggs (Witkowska, 1981, 1982).

Remodelling of thymocyte nuclei consists of a series of morphological changes associated with decondensation and swelling. Thymocytes are cells that are active in DNA synthesis (as rapidly proliferating cells), but presumably non-active in RNA synthesis. Formation of nucleolus-like structures (or nucleoli) in th-nuclei introduced into eggs is reminiscent of nucleologenesis in dormant erythrocyte nuclei following cell fusion with human somatic cells (Ringertz & Savage, 1976; Hernandez-Verdun & Bouteille, 1979). Electron microscopic studies of remodelled th-nuclei are in progress.

Swelling of thymocyte nuclei (shown by the increase in the nuclear volume up to 200-fold) appears to be a general reaction of foreign nuclei transplanted into activated eggs (amphibians, see Subtelny & Bradt, 1963; Graham, Arms & Gurdon, 1966). Nuclear-transplantation and cell-fusion experiments have demonstrated that nuclear swelling, associated with decondensation of chromatin, is a prerequisite for nuclear reprogramming (for reviews see Ringertz & Savage, 1976; DiBerardino, 1980). However, there are also data suggesting that swelling, *per se*, need not unequivocally indicate active DNA or RNA synthesis (Merriam, 1969; Ringertz & Savage, 1976). Thus, the cytological approach alone, as used in this study, does not provide sufficient evidence to consider morphological remodelling of th-nuclei as proof of their synthetic reprogramming towards the pronucleus-like type.

In summary, we conclude that of the two main experimental variants studied, i.e. oocytes activated after PEG treatment and oocytes submitted to PEG treatment after activation, the first one ensures full remodelling of single th-nuclei provided that the time between PEG and activation is not prolonged. In the second variant, conditions ensuring nuclear remodelling are still retained for about 1 h.

Recent experiments of Hoffner & DiBerardino (1980), Leonard *et al.* (1982) and DiBerardino & Hoffner (1983) on amphibian oocytes have demonstrated that passage of embryonic nuclei and dormant somatic nuclei through the cytoplasm of maturing oocytes increases the ability of these nuclei to direct the development of enucleated eggs up to larval stages. It seems likely that in mammals also the optimal conditions for nuclear transfer are created when the introduction of foreign nuclei precedes oocyte activation. It is conceivable, however, that due to an extremely long first cell

cycle, the cytoplasm of an activated mammalian egg may also be potent to reprogramme foreign nuclei even if they do not pass through a chromatin-condensation stage.

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