Reprod. Nutr. Dev. (1989) 29, 559-568

Original article

In vitro culture of bovine egg fertilized either *in vivo* or *in vitro*

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(3rd Franco-Czechoslovak Meeting, INRA, Jouy-en-Josas, 13–14 December 1988)

Summary — Three-quarters of *in vivo* and one-third of *in vitro* fertilized bovine eggs reached blastocyst stage when cultured on tubal cell monolayers (TCM), but no hatching occurred in B2 medium supplemented with estrous cow serum. When after 3 days of culture on TCM, morulae were transferred on endometrial cell monolayers (UCM), the same proportion of blastocysts was obtained and one-third of them hatched.

Histological studies of hatched blastocysts showed that the number of inner cells was significantly lower than in hatched blastocysts recovered *in vivo* 8—8.5 days after ovulation. Moreover, the number of pycnotic cells was higher than normal, although mitosis were present. On the contrary, there was no difference in either the number or the appearance of trophoblastic cells between blastocysts obtained *in vitro* and *in vivo*. The addition of transforming growth factor (TGF-β) to either TCM or UCM co-cultures at the very beginning of blastocyst formation specifically stimulated growth of the inner cell mass (ICM). The number of cells at hatching was about double (120) and significantly higher than that found in 8—8.5-day blastocysts *in vivo*. Moreover, hatching percentages were similar to the controls, even when eggs were cultured for 8 days only on TCM. However the proportion of pycnotic cells remained higher than normal, although many mitotic cells were unevenly distributed in ICM) *In vivo* during hatching, there were always pycnotic cells in ICM, but their number was limited and approximately similar to the number of mitosis. The uterine factors which control both mitosis and pycnosis in ICM remain to be discovered.

culture — bovine blastocyst — hatching — TGF-β

Résumé — **Culture *in vitro* de l'œuf de vache fécondé soit *in vitro* soit *in vivo*.** *Il est possible de contrôler rapidement la réussite de la fécondation* in vitro, *l'efficacité du transfert de gènes ou celle du clonage, à condition, de cultiver l'œuf au moins jusqu'au stade blastocyste éclos. Ceci est particulièrement important pour les gros mammifères dont le coût ne permet pas de tester tous les essais par des transferts dans des femelles receveuses. Par ailleurs, chez les bovins, le transfert par voie non chirurgicale ne pouvant se faire avant le 6—7e j après l'ovulation il importe de disposer de blastocystes et non simplement d'œufs aux premiers stades de la fécondation comme chez les primates. Nous avons donc cherché à obtenir par culture des blastocystes pouvant être considérés comme normaux par comparaison avec des blastocystes récupérés* in vivo *7 j et 8,5 j après la fécondation (BI-7 et BI-8,5). La culture sur tapis de cellules tubaires de vache (TCM) permet le déve-*

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loppement jusqu'au stade blastocyste expansé mais jamais jusqu'à l'éclosion. A partir d'œufs cultivés 3 j sur TCM, puis 4-5 j sur tapis de cellules utérines (UCM), nous avons obtenu respectivement 31% d'éclosions pour les œufs fécondés in vivo *et 37% pour les œufs fécondés* in vitro, *en mimant les conditions naturelles; ces valeurs sont significativement inférieures à celle observée* in vivo *(70%) dans des délais semblables. De plus, le nombre de cellules du bouton embryonnaire (ICM) de ces blastocystes éclos est significativement plus petit que celui des BI-8,5 (63 contre 87, Fig. 1a), alors que le nombre de cellules du trophoblaste n'est pas significativement différent (Fig. 1b). Enfin, bien que des mitoses soient régulièrement présentes, le nombre de cellules picnotiques est significativement plus élevé que chez les BI-8,5. Le rôle joué par le TGF-β (transforming growth factor-β) dans la différenciation de l'embryon de xénope, nous a conduit à rechercher si la présence de ce facteur de croissance dans le milieu de culture pouvait augmenter le nombre de cellules de l'ICM et réduire le taux de cellules picnotiques. Le TGF-β entraîne effectivement une augmentation hautement significative du nombre de cellules (120 contre 87). Le nombre de mitoses est également plus élevé, bien que non significativement (Fig. 2a). Malgré cela le nombre de cellules picnotiques demeure significativement plus important que chez les BI-8,5 (Tableaux III et IV). Par ailleurs, la présence de TGF-β élève le taux d'éclosion à des valeurs identiques à celle des BI-8,5 et permet l'éclosion sur TCM sans passage sur UCM et ceci à un taux identique à celui des témoins BI-8,5. Ainsi TGF-β permet d'augmenter spécifiquement le nombre de cellules de l'ICM, même au delà de la valeur normale, alors qu'il est sans effet significatif sur le trophoblaste. De plus, il permet un taux d'éclosion* in vitro *identique à celui observé* in vivo *8,5 jours après la fécondation. La juxtaposition dans l'ICM de mitoses et de cellules pycnotiques mais en nombre plus élevé que dans les BI 7 et 8,5 montre que le milieu utérin règle la taille de l'ICM en limitant le nombre de cellules qui dégénèrent, ce que nous n'avons pas réussi à obtenir dans notre système de culture.*

culture — blastocyste — vache — éclosion — TGF-β

INTRODUCTION

It has been shown that *in vivo* fertilized ovine and caprine eggs are unable to cleave in culture beyond the 8—12-cell stage (Wintenberger *et al.*, 1954). The same observation has been made on bovine egg (Thibault, 1966). Many attempts to stimulate further cleavage by enriching classical culture media with ovine, bovine, equine or fetal calf blood serum have been unsuccessful. A discovery by Gandolfi & Moor (1987) showed that the 8-cell stage block did not occur if sheep *in vivo*-fertilized eggs were cultured on epithelial tubal cell monolayers, or even on fibroblasts. After such co-cultures, morulae and blastocysts were obtained and lambs were born from transferred blastocysts. This procedure has been extended to bovine egg (see Rexroad, 1989). Granulosa cells also seem able to support complete development (Goto *et al.*, 1988). However, the percentage of eggs reaching the blastocyst stage is low (15%, Goto *et al.*, 1988). The same conclusion can be inferred from the fact that, in many papers, morulae and blastocysts are presented together. In cattle, non-surgical embryo transfer by the cervical route cannot be performed before day 6 or 7. At this time the embryo has reached the blastocyst stage. Thus in view of practical application, *in vitro* fertilized eggs must be cultured beyond the morula stage. An adequate culture system may also offer the possibility to check the presence and possibly the early expression of genes tranferred at one-cell stage and the efficacy of cloning procedures, thereby avoiding expensive transfer to recipient females. It was the purpose of this study to obtain normal hatched blastocysts in culture from *in vivo* or *in vitro* fertilized bovine eggs.

MATERIAL AND METHODS

Cow and heifers of Friesian or Charolais breed were synchronized according to Eldsen *et al.* (1978) by "Estrumate" (Coopers, France) and superovulated by a partially purified pFSH preparation containing ≈ 10% of LH. Final follicular maturation and ovulation were synchronized by injection of the equivalent of 2 mg of pure pLH at the beginning of estrus. These hormones were prepared and generously supplied by Combarnous (INRA, CNRS). Samples of frozen semen from the same bull were used for *in vivo* and *in vitro* inseminations.

Oocytes used in *in vitro* fertilization were recovered 24—26 h after LH injection by puncture of the largest follicles. Delay between slaughter and puncture was 15—30 min. Oocytes encompassed in expanded cumulus were cultured in B2 medium (INRA, Ménézo) +10% of estrus cow serum (ECS) for 4—6 h before *in vitro* insemination. *In vivo* fertilized eggs were recovered either a few hours after ovulation or 24 h later by flushing the tubes with PBS within 30 min after slaughter. The eggs were rapidly transferred to the selected medium. All manipulations were performed in sterile rooms maintained at 32—35 °C and at 80—90% humidity.

In vitro fertilization procedure has been described by Marquant-Le Guienne *et al.* (1989). *In vitro* fertilized eggs were freed of corona cells and attached sperms 18 h after insemination in B2 medium supplemented with 10% ECS and then transferred to tubal cell monolayers. *In vivo* fertilized eggs recovered either a few hours after fertilization or 30 h later were cultured under the same conditions.

Blastocysts were recovered *in vivo* by the cervical route 7 or 8.5 days after ovulation according to Ozil *et al.* (1980). Ovulation has been estimated to occur 30 h after the onset of estrus (Thibault, 1967).

Tubal cell monolayers were prepared as follows : 1 ml of HBSS (without Ca^{2+} and Mg^{2+}) containing 0,46 mg of trypsin (activity 3.6 U/mg, SERVA) + 1.4 mg of collagenase (0.7—0.8 U/mg, SERVA)) was infused into the lumen of each tube ligated at both ends. Thirty min later, the content was flushed with 1 ml of cow or calf serum and centrifuged for 15 min at 300 g. The supernatant was discarded and small pieces of tubal epithelium were gently resuspended in 0,5 ml of B2 medium supplemented with 10% of ECS. Generally 100 µl of this suspension was distributed in Nunc or Falcon plates, each well containing 350 µl of the same medium. Non-adhering cells or debris were removed 24 h later and the medium was renewed every 2 or 3 days.

Uterine cell monolayers were also prepared from cows slaughtered for oocytes or fertilized egg collection on day 0 or day 1. This is not the optimal time during the estrus cycle for uterine cell viability (Fortier *et al.*, 1988) and plating occurred irregularly. Therefore two techniques were used simultaneously. After ligation of both ends of the uterine horn, 10 ml of HBSS + trypsin and collagenase were infused into the horn through the myometrium in sterile conditions. One hour later, HBSS was removed by aspiration and the horn was opened. Small pieces of tissue were obtained by gently scraping the epithelium. Cow or calf serum was then added to stop proteolytic activity. The biggest fragments were removed and the suspension was centrifuged as described for tubal cell preparation. The second uterine horn was opened and shreds of epithelium were removed, put in HBSS + enzymes (trypsin + collagenase or collagenase alone) and shaken for 10—15 min. Cow or calf serum was added and the procedure was carried out as previously mentioned. All manipulations were performed at 25—27 °C in sterile conditions. As uterine cells adhere slowly, non-adhering cells and debris were completely removed after 48 h or even 72 h. After 3 days of culture, B2 + ECS was replaced by B2 + blood serum of day 6 cow (D6 CS). No care was taken to isolate the epithelial and stromal cells since it has been shown that stromal cells are absolutely necessary to the formation of progesterone receptors in epithelial cells in response to estradiol (Inaba *et al.*, 1988). The two-type cell ratio did not change whatever the culture time.

Under our culture conditions (B2 medium + cow serum, under air + 5% CO_2, pH 7.4), the tubal and uterine cells, after covering the bottom of the wells, stopped dividing and remained able to support blastocyst formation for many weeks and sometimes many months. Therefore we only used primary cultures.

In some experiments, transforming growth factor β (TGF-β), (BTI, MA, USA) was added at

a concentration of 0.3 ng/ml to the culture at the early beginning of the formation of blastocoele cavity; thereafter 0.1 ng/ml was added every day up to the day of fixation.

Eggs, morulae and blastocysts were fixed in ethanol—acetic acid (3/1) for 1—2 days, then post-fixed in Bouin Hollande fixative for 24 h, double-embedded in Agar—paraffin, cut into serial sections 15 µm thick, and stained with hematoxylin—eosin. The nuclear state (normal, pycnotic, interphasic or mitotic was assessed and the number of cells determined by microscopical examination (480 x). The nuclei of the inner cell mass (ICM) were small and round, whereas those of trophoblast were flat and bigger. This criterion was used to determine the number of cells of trophoblast and ICM. The Fisher exact test was used in studies on hatching rates. Comparisons of the numbers of cells, numbers of mitotic and pycnotic cells between each experimental group and their controls were carried out by the two sample *t*-tests when variances were equal or by Satterthwhite's approximation when variances were unequal (SAS/Stat Guide, 1987). Absolute numbers of mitoses and pycnoses were analyzed after square root transformation (*V* number + 0.5) (Steel and Torrie, 1980).

RESULTS

When *in vivo* or *in vitro* fertilized eggs were cultured in B2 medium supplemented by fetal calf serum, a high proportion of eggs cleaved regularly (Table I) but none of them cleaved beyond the 8—12-cell stage. When *in vivo* or *in vitro* fertilized eggs were cultured on tubal cell monolayer, they cleaved regularly and 73% of the morulae formed blastocysts (Table II). About fifty percent of them were expanded after 8 days of culture, but none of them hatched (even after 11 days).

As it is known that in non-primate mammals, fertilized eggs experimentally maintained in the tubes degenerate at blastocyst stage, we tried to mimic the natural *in vivo* situation by transferring morulae on uterine cell monolayers (UCM) after 3 days of culture on tubal cell monolayers (TCM). In these conditions, 68% of the morula developed to blastocysts, and ≈ 8 days after fertilization 31% of these blastocysts hatched (Table II). When *in vitro* fertilized eggs were cultured under the same condi-

Table I. *In vitro* fertilization and cleavage of bovine oocytes matured either *in vitro* or *in vivo*.

Oocyte maturation	*Fertilization*		*Cleavage*		*> 8—12 cells after 96 h in B2 + FCS**
	No.	*%*	*No.*	*%*	
In vitro 26—30 h B2 + ECS ** and granulosa cells	100/128	78	64/74***	86	not studied
In vivo 24 h + *in vitro* 4—6 h	28/34	82	27/28	96	0/27

* B2 + FCS : B2 medium + fetal calf serum.
** B2 + ECS : B2 medium + estrus cow serum.
*** some fertilized eggs have been fixed.

Table II. Culture of *in vivo* or *in vitro* fertilized bovine eggs on tubal cell monolayers (TCM) or TCM and uterine cell monolayers (UCM) with or without transforming growth factor β (TGF-β)

Culture systems	*Duration in days*	*Blastocyst morula*		*Hatched blastocyst*	
		No.	*%*	*No.*	*%*
TCM (1)	≥ 8 d	25/34	73	0 / 25	0 a
TCM + UCM (1)	> 7 d	54/89	68	16 / 51	31 b^1
TCM + UCM (2)	8 d	10/30	33	3 / 8	37 b^2
TCM (1) + TGF—β (2d)	8 d	8/15	53	7 / 8	87 c
TCM + UCM (1) + TGF-β (2d)	≥ 7.5 d	16/17	94	10 / 16	62 d
In vivo blastocysts	8.5 day			7 / 10	70 e

(1) Fertilization *in vivo*; (2) fertilization *in vitro*.
b^1, b^2 *vs* e, $P < 0.03$; b^1 *vs* d, $P < 0.04$; a *vs* c, $P < 0.01$.

tions, a lower proportion of morulae reached the blastocyst stage (33%) but a similar percentage of hatching was observed (37%, Table II). Thus the passage of morulae from TCM to UCM allowed hatching. Hatching followed a time schedule similar to that *in vivo* 8—8.5 days after fertilization (Table III). However, histological studies showed that *in vitro* hatched blastocysts had a significantly lower number of cells in ICM than those found in *in vivo* hatched blastocysts ($P < 0.02$, Fig. 1a and Table IV). Moroever, the average number of pycnotic cells in ICM was much higher ($P < 0.01$), although mitoses were present in ICM as well as in the trophoblast (Tables III and IV).

The relatively low number of cells in ICM and the simultaneous presence of mitotic and degenerating cells suggests that our culture conditions did not provide any factors which could control the growth and differentiation of ICM, whereas the trophoblast remained in every case perfectly healthy. This hypothesis has been tested by adding to the culture medium transforming growth factor β (TGF-β) which is known to play a key role in the early differentiation of *Xenopus* embryo (Kimelman & Kirschner, 1987; Weeks & Melton, 1987; Harland, 1988). TGF-β was added to the culture medium of both tubal and uterine cells when the most advanced morulae were reaching the very early blastocyst stage. Thus *in vivo* fertilized eggs either remained on tubal cells for 8 days (TGF-β being present during the last 2 days) or were transferred to uterine cells at day 4 (TGF-β being added during the last 2 or 3 days of culture). TGF-β significantly enhanced the hatching rate of TCM-UCM embryos (62 *vs* 31; $P < 0.04$, Table II). Moreover, hatching occurred when the eggs remained on TCM (87% vs 0 %; $P < 0.01$, Table II). In these *in vitro* systems hatching occurred as *in vivo*, *i.e.* ≈ 8 days after fertilization.

Table III. Histological studies of bovine blastocysts collected *in vivo* 7 or 8.5 days after fertilization.

Blastocyst	*Total No. cells*	*Trophoblast No. cells*	*ICM No. cells*	*Ratio ICM/total*	*Inner cell mass*	
					mitosis	*pycnosis*
7-day old expanded (14)**	216 173-255	134 102-184	82 55-110	0.38	6 1-14	5 1-14
8.5-day old expanded (3)	288 272-304	201 186-222	87 82-93	0.30	2 1-3	0
8.5-day old hatched (7)	251 197-309	164 134-213	87 62-100	0.35	4 2-7	3 0-8

* Fertilization has been estimated to occur 30 h after the onset of estrus (Thibault, 1967).
** (...) Number of blastocysts studied.

Table IV. Comparison of cell number and cell quality of hatched blastocysts obtained after # 8 days of culture in different conditions.

Culture system	*Cell No.*			*Ratio*	*ICM*	
	Total	*trophoblast*	*ICM*	*ICM/Total*	*mitosis*	*pycnosis*
TCM—UCM (1)	197 (121—316)	134 (76—223)	63 (24—110)	0.32	3 (0—16)	17 a (0—46)
TCM—UCM (2)	200 (189—220)	139 (131—153)	61 (57—67)	0.31	7 (5—8)	5 (3—6)
TCM + TGF-β (1)	256 (230—284)	139 (115—176)	118 (102—137)	0.46	5 (3—7)	39 a (23—57)
TCM—UCM + TGF-β (1)	300 (214—411)	191 (122—263)	120 (60—162)	0.38	8 (0—17)	32 a (9—54)

(1) *In vivo* fertilization; (2) *in vitro* fertilization; (...) range.
a : significantly higher than *in vivo* control ($P < 0.01$, Table III).
Number of embryos studied are given in Figure 1.

Were these blastocysts similar to those recovered* in vivo *from superovulated cows 8-8.5 days after fertilization ?

When blastocysts were cultured on TCM—UCM plus TGF-β the numbers of cells in ICM were significantly higher ($P < 0.01$) than in blastocysts cultured without this factor (Fig. 1a, Tables III and IV). In hatched blastocysts obtained when TGF-β was added to TCM culture, the numbers of cells in ICM were significantly higher than in *in vivo* controls ($P < 0.01$, Tables III and IV, Fig. 1a). No comparison could be made with blastocysts obtained on TCM alone since no hatching was observed in that situation. These results indicate that TGF-β always stimulated mitotic activity. However, the numbers of pycnotic cells in ICM remained significantly higher than in *in vivo* controls ($P < 0.01$; Tables III and IV).

When comparing the numbers of mitotic and pycnotic cells in ICMs of *in vitro* produced blastocysts (Fig. 2a, 2b) it is evident first that very few of them were in the range values of *in vivo* hatched blastocysts; and second, that *in vitro* there was a great variation in histological parameters from one blastocyst to the other. These observations indicate that very few blastocysts produced *in vitro* would be able to develop up to term.

As shown in Table III, the growth of ICM *in vivo* between day 7 and day 8.5 was limited (87 cells *vs* 82 cells) and lower than in trophoblast (6% *vs* 22%). A few mitoses were always observed (2 to 7), but pycnotic cells were also present (0 to 8, Table III).

A differential rate of growth between trophoblast and ICM during expansion and hatching seemed to occur *in vivo*; the ratios between ICM and total cell number were respectively 0.38 and 0.35 in blastocysts recovered in the uterus at day 7 and day 8.5 (Table III). The ratios of hatched blastocysts obtained *in vitro* were different; they were lower in TCM—UCM-hatched blastocysts (0.32) and higher in the two TGF-β groups (0.46 and 0.40) (Table IV). As in all the experimental groups, the numbers of trophoblastic cells did not differ significantly from the control (Fig. 1b), the ratio differences between *in vivo* and *in vitro* blastocysts mainly depended on ICM development (compare Table III and IV). Thus the low ratio (0.32) in TCM—UCM hatched blastocyst was due to an underdevelopment of ICM and the high ratios,

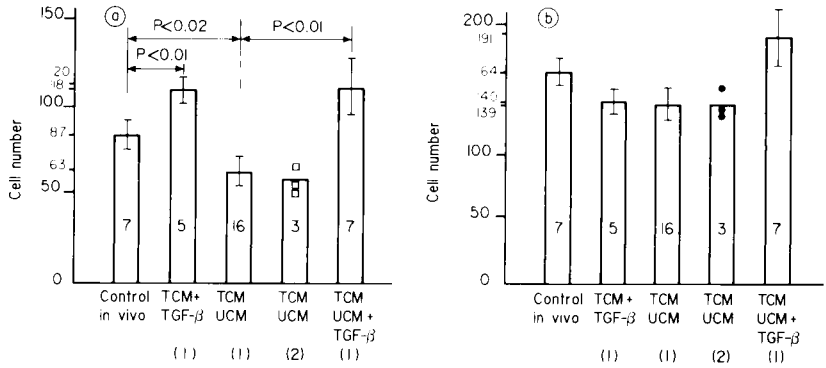

Fig. 1. Number of cells in inner cell mass (**a**) and in trophoblast (**b**). Number of embryos are indicated in the columns. **Control** = blastocysts flushed 8.5 days after fertilization; **TCM** = tubal cell monolayer; **UCM** = uterine cell monolayer. **TCM–UCM** = eggs were cultured 3 days on TCM then on UCM. (1) *In vivo* fertilized eggs; (2) *in vitro* fertilized eggs.

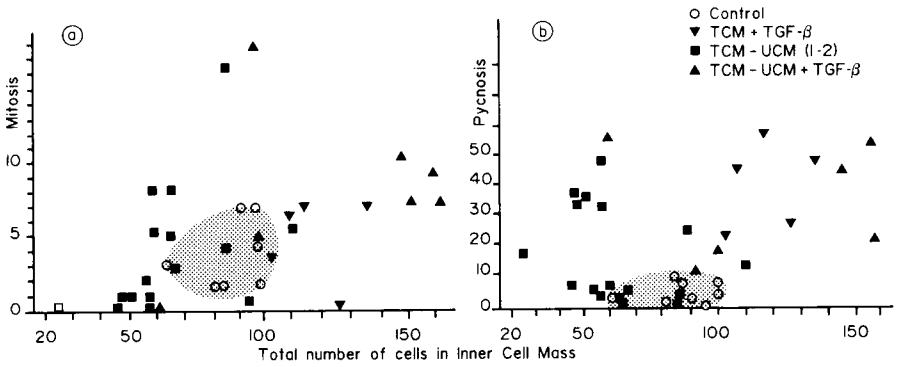

Fig. 2. Number of mitotic cells (**a**) and pycnotic cells (**b**) in inner cell mass of *in vivo* and *in vitro* hatched blastocysts. In X-axis are the number of cells in ICM. Numbers from *in vivo* blastocysts are included in shaded area.

when TGF-β was present, resulted from a dramatic increase of cell multiplication only in ICM.

DISCUSSION

The culture of bovine fertilized eggs on tubal cell monolayers in B2 medium supplemented with estrus cow serum allowed a high rate of blastocyst formation. However, no hatching occurred although the total number of cells was in the range of *in vivo* hatched blastocysts (172—262 *vs* 173—255; data not shown). On the contrary, when cultured on uterine cell monolayers after 3-day culture on TCM, one-third of the blastocysts hatched, although the number of cells of the whole blastocysts and of ICMs as well as their ratios were lower than in *in vivo* controls. There is no similar information on any species in the literature. Kajihara *et al.* (1988) have only given the total number of cells in hatching + hatched bovine blastocysts obtained after 8 to 12 days of culture on granulosa cells of *in vitro* fertilized eggs. Moroever, the average number of cells was surprisingly low (mean = 128, range 22 (?)—250) for this stage of development.

A differential rate of growth between trophoblast and ICM during expansion and hatching has been already mentioned by Papaionnaou & Ebert (1988) in pig embryo, the ratio between ICM and total cell number decreasing after hatching from 0.24 to 0.15.

TGF-β has a wide range of effects (see Rizzino, 1988). On cell growth TGF-β seemed to potentiate or suppress the growth-stimulatory activity of other growth factors. Mesoderm differentiation of *Xenopus* embryo depends on a synergistic action of TGF-β and fibroblast growth factor (FGF) (Kimelman & Kirschner, 1987). In mammals, it has been recently reported that mouse blastocyst cultured *in vitro* produces a growth factor which is immunologically related to FGF (Rizzino, 1988) but the presence of TGF-β in this species has been only studied in later stages of development (Heine *et al.*, 1987). Nothing is known in other mammals.

In our experiments, it is clear that TGF-β mainly stimulated mitotic activity in ICM. This effect might be indirect, *via* a stimulation of other growth factors from tubal and uterine cells. Whatever the mitogenic growth factor involved, this response seems to indicate that growth factor receptors are present in ICM. *In vivo* during the hatching period, the uterine milieu probably stimulates the multiplication of inner cells by growth factors and simultaneously dampers ICM development by allowing a limited rate of pycnosis. This double mechanism is evidenced in mouse blastocysts during delayed implantation; in this situation, the number of inner cells does not increase, or even decreases, although DNA synthesis is maintained in ICM for at least a few days (Given, 1988). Unfortunately uterine factors involved in the arrest of blastocyst development in delayed implantation have not been identified yet. In our culture system we could not stimulate the multiplication of inner cells and simultaneously limit the number of pycnotic cells to a normal rate. We can speculate that our system could not support metabolic exchanges in ICM or could not provide specific uterine factors, for instance from endometrial glands. These hypotheses are now under investigation.

It is generally assumed that TGF-β plays a role in cell differentiation via its effects on the extracellular matrix; however, in our experiments we only observed a mitogenic effect and endoderm differentiation was not enhanced.

ACKNOWLEDGMENTS

We would like to thank P. Cochaud, B. Moret and the farm staff of Bressonvilliers and Jouy-en-Josas for assistance with the management of animals.

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