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An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*

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Summary. One-cell CF-1 × B_6SJLF_1/J embryos, which usually exhibit a 2-cell block to development *in vitro*, have been cultured to the blastocyst stage using CZB medium and a glucose washing procedure. CZB medium is a further modification of modified BMOC-2 containing an increased lactate/pyruvate ratio of 116, 1 mM-glutamine and 0·1 mM-EDTA but lacking glucose. Continuous culture of one-cell embryos in CZB medium allowed 83% of embryos to develop beyond the 2-cell stage of which 63% were morulae at 72 h of culture, but blastocysts did not develop. However, washing embryos into CZB medium containing glucose after 48 h of culture (3–4-cell stage) was sufficient to allow development to proceed, with 48% of embryos reaching the blastocyst stage by 96 h of culture. Exposure of embryos to glucose was only necessary from the 3–4-cell stage through the early morula stage since washing back into medium CZB without glucose at 72 h of culture still promoted the development of 50% of embryos to the blastocyst stage. The presence of glucose in this medium for the first 48 h of culture (1-cell to 4-cell stage) was detrimental to embryo development. Glutamine, however, exerted a beneficial effect on embryo development from the 1-cell to the 4-cell stage although its presence was not required for development to proceed during the final 48 h of culture. Blastocysts which developed under optimum conditions contained an average of 33·7 total cells. The in-vitro development of 1-cell embryos beyond the 2-cell stage in response to the removal of glucose and the addition of glutamine to the culture medium suggests that glucose may block some essential metabolic process, and that glutamine may be a preferred energy substrate during early development for these mouse embryos.

Keywords: mouse; embryo; 2-cell block; glutamine; glucose

Introduction

The culture of 2-cell mouse embryos to the blastocyst stage can now be performed with consistency in a variety of chemically defined media containing lactate alone (Whitten, 1957; Brinster, 1963) or lactate and pyruvate (Brinster, 1965a) as energy sources. These blastocysts are capable of implantation and fetal development upon transfer to recipient pseudopregnant females (Biggers *et al.*, 1965).

The culture of 1-cell mouse embryos to the blastocyst stage has met with more limited success. With the exception of some inbred and F1 strains (Whitten & Biggers, 1968; Kaufman & Sachs, 1976; Goddard & Pratt, 1983), 1-cell mouse embryos exhibit what has been termed a '2-cell block' to development *in vitro*, i.e. the 1-cell embryos undergo a single cleavage division *in vitro* and are arrested at the 2-cell stage. In experiments using reciprocal crosses between blocking and non-blocking strains, Goddard & Pratt (1983) have demonstrated that this in-vitro 2-cell block is a maternally regulated phenomenon. By transferring cytoplasm from a non-blocking embryo to a

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blocking embryo, Muggleton-Harris *et al.* (1982) and Pratt & Muggleton-Harris (1988) have shown that the block is mediated by a cytoplasmic component which may be lacking in embryos from blocking strains.

The ability of 1-cell mouse embryos to develop *in vitro* has been shown to be a function of mouse strain (Whitten & Biggers, 1968; Kaufman & Sachs, 1976; Abramczuk *et al.*, 1977), medium components (Biggers *et al.*, 1967; Whittingham & Biggers, 1967; Cross & Brinster, 1973; Abramczuk *et al.*, 1977; Loutradis *et al.*, 1987) and culture conditions (Quinn & Harlow, 1978).

Two factors reported independently to be beneficial for culture of some blocking strains of embryos are: a lactate/pyruvate ratio approaching 120 (Cross & Brinster, 1973) and the addition of EDTA to the culture medium (Abramczuk *et al.*, 1977). However, there is considerable variation in the success of these culture modifications in different laboratories.

The mouse is not the only species which exhibits a block to development *in vitro* as hamster, rat and pig all exhibit a block to in-vitro development at various stages. For example, golden hamster embryos block *in vitro* at both the 2- and 4-cell stage and only 2% of 8-cell embryos develop to blastocysts (Bavister *et al.*, 1983). However, supplementation of the basic culture medium with a group of four amino acids increased the development of 8-cell hamster embryos to blastocysts to 36% (Bavister *et al.*, 1983). Carney & Bavister (1987) showed that, of the four amino acids in this culture medium, glutamine was the most efficient at improving development.

In this study we have attempted to develop a completely defined medium which will consistently allow culture of 1-cell embryos from mice which exhibit a block *in vitro* to the blastocyst stage. We have selected the CF-1 mouse for this study because it blocks at the 2-cell stage in all media tested in our laboratory although Dandekar & Glass (1987) have reported the culture of CF-1 embryos to the blastocyst stage in a medium containing human serum. Furthermore, it is highly economic for scientists to use this CF-1 strain since it produces an average of 40–50 embryos per female.

Materials and Methods

Embryo collection. CF-1 female mice (Harlan Sprague Dawley, Colony 202, Indianapolis, IN, USA) were superovulated with intraperitoneal injections of 10 i.u. PMSG (Calbiochem, La Jolla, CA, USA) followed 48 h later by 5 i.u. hCG (Organon, W. Orange, NJ, USA). Females were mated overnight with B6SJLF1/J males (an F1 hybrid from C57BL/6J × SJL/J: Jackson Laboratories, Bar Harbor, ME, USA). One-cell embryos were flushed from excised oviducts at 25–27 h after hCG (Day 1) in Hank's balanced salt solution containing 4 mg bovine serum albumin/ml (Cat. No. 0140, Armour Pharmaceutical Co. Kankakee, IL, USA) (HBSS + BSA).

When necessary, cumulus cells were removed from fertilized ova by hyaluronidase treatment (300 units/ml in phosphate-buffered saline (PBS) containing 1% polyvinylpyrrolidone; Sigma Chemical Co., St Louis, MO, USA) and embryos were then washed through three drops of HBSS + BSA. Embryos were placed in a holding dish containing 50 µl drops of culture medium overlaid with Fisher 121 paraffin oil (Fisher, Medford, MA, USA) which was washed before use with two changes of complete CZB medium for 96 h with stirring (10 ml medium per 100 ml oil). All collections were performed in a darkened room as Schumacher & Fischer (1988) suggested that as little as 1 h of exposure to light is significantly harmful to Day-1 rabbit embryo development. All solutions, dishes and instruments were maintained at 37°C before use.

Culture media. Embryos were cultured in four basic media: a modified version of Whittingham's (1971) original Medium 16 (M16), modified Whitten's medium (WM) (Hoppe, 1985), modified Earle's BSS (EBSS) with EDTA (Dr Ann Kiessling, personal communication) and a modified BMOC-2 medium (Ebert & Brinster, 1983) which has been further modified and designated CZB medium. Formulations for these media are shown in Table 1. Media variations were based on changes in these formulae. Glutamine was added to media from a fresh 100 mM-glutamine stock immediately before use. Medium was prepared on the basis of weight in T25 or T75 polystyrene tissue culture bottles (Corning, Medfield, MA, USA) using endotoxin-free, tissue culture-grade water (Sigma Chemical Co., Cat. No. W3500). Media were filter-sterilized through 0·22 µm Millipore filters (Millipore Corp., Bedford, MA, USA) in Nalgene reusable filter holders (Cat. No. 300-4000). Media were gassed with 5% CO_2/5% O_2/90% N_2 and stored at 4°C. Fresh media were prepared every 2 weeks. Osmolarity of all media was tested by freezing point depression and ranged from 274 to 295 mosmol which is within the acceptable range of osmolarities determined by Brinster (1965b) for development of 2-cell embryos.

Culture procedures. Media were pipetted in 50 µl drops into Falcon 60-mm Petri dishes (Falcon Cat. No. 1007, Beckton Dickinson, Lincoln Park, NJ, USA) and overlaid with medium-washed Fisher 121 paraffin oil. Media drops in

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Table 1. Media formulation

Component†	Concentration (mM)				
	Modified M16	Modified WM	EBSS + EDTA*	Modified BMOC-2	CZB§
NaCl	94·62	109·51	117·00	88·98	81·62
KCl	4·83	4·83	5·40	4·83	4·83
KH_2PO_4	1·18	1·18	—	1·18	1·18
$MgSO_4.7H_2O$	1·18	1·18	0·80	1·18	1·18
$NaHCO_3$	25·00	22·62	22·00	25·12	25·12
$NaH_2PO_4.H_2O$	—	—	1·00	—	—
$CaCl_2.2H_2O$	1·70	—	1·80	1·70	1·70
D-Glucose	5·55	5·55	5·55	5·55	—
Sodium lactate‡	22·00	—	26·17	29·90	31·30
Calcium lactate	—	4·86	0·17	—	—
Sodium pyruvate	0·55	0·27	0·30	0·27	0·27
EDTA (disodium salt)	—	—	0·01	0·11	0·11
Glutamine	—	—	—	—	1·00
BSA (mg/ml)	4·00	3·00	4·00	5·00	5·00
Sodium penicillin G (U/ml)	100·00	121·00 (K salt)	133·00	100·00	100·00
Streptomycin (mg//ml)	0·50	0·50	—	0·70	0·70
Phenol red (mg/ml)	—	—	0·01	—	—
Lactate/pyruvate ratio	40·00	17·89	87·70	110·74	115·92

*EBSS was purchased from GIBCO (Cat. No. 450–1100 Grand Island, NY, USA) and supplemented with sodium bicarbonate, sodium lactate, calcium lactate, EDTA, pyruvate and BSA (Dr Ann Kiessling, personal communication).

†KCl and $NaHCO_3$ were Mallinckrodt Chemicals (Mallinckrodt Inc., Paris, KY, USA). BSA was obtained from Armour Pharmaceutical Co. (Kankakee, IL, USA). All other components were obtained from Sigma Chemical Co. (St Louis, MO, USA).

‡Concentration based on using a 60% solution of DL-sodium lactate on a w/w basis (Sigma Chemical Co., St Louis, MO, USA).

§CZB with glucose contains 5·55 mM-glucose.

culture plates were equilibrated with 5% CO_2/5% O_2/90% N_2 in a sealed chamber (Billips Rothenberg, Del Mar, CA, USA) at 37°C for 4–5 h before use. One-cell embryos were removed from the holding dish and washed through one drop of test medium into a final culture drop. A culture drop of 50 µl contained a maximum of 25 embryos. Cultures were incubated in sealed culture chambers gassed with 5% CO_2/5% O_2/90% N_2 at 37°C. In experiments in which washing was required, embryos were removed from the culture chamber at 24 (Day 2), 48 (Day 3) or 72 (Day 4) h of culture and transferred through a wash drop of fresh medium into a culture drop of fresh medium as indicated and returned to the culture chamber. In some experiments, glucose (from a 100 mg/ml stock) was injected directly into the original culture drop at 48 h of culture (Day 3). Cultures were terminated at 96 h (Day 4, 121–123 h after hCG). Embryos were scored for developmental stage and abnormalities on Days 4 and 5 and were then fixed on Day 5 in 4% paraformaldehyde in PBS, washed into PBS and stained with Hoechst 33258 (10 µg/ml in HBSS + BSA, Cat. No. B2883 Sigma Chemical Co., St Louis, MO, USA) to label the nuclei fluorescently. Nuclei were counted on a Nikon Diaphot inverted phase-contrast microscope with fluorescence attachment under u.v. illumination. Average nuclei per morula and per blastocyst were calculated. Nuclei of abnormal embryos were not counted as many had undergone significant degeneration.

Statistical analysis. To control for variation in development between animals, embryos from each animal were randomly distributed among all culture samples in a given experiment unless otherwise noted. Each experimental protocol represents a minimum of 2 replicates performed on different days or by different people. For all experiments, a complete set of samples was run simultaneously (e.g. for each matrix replicate all 4 samples were run at the same time). Statistical analysis of experiments was performed with an NCSS software package (Number Cruncher Statistical System, NCSS, Kaysville, UT, USA) using a standard arcsin transformation (Sokal & Rohlf, 1981) of the data and analysis of variance or Student's *t* test. Statistical analysis of morula and blastocyst nuclear counts was performed on the non-transformed counts using Student's *t* test.

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Results

In initial experiments, when 1-cell CF1 × B6SJF1/J embryos were cultured in Medium M16, WM or EBSS with EDTA for 96 h (Table 2), embryo development was generally blocked at the 2-cell stage on Day 4 with some proceeding further but not beyond the 8-cell stage. Other preliminary experiments (data not shown) indicated that addition of 1 mM-glutamine to or the elimination of glucose from Medium M16 would allow 7–34% of 1-cell embryos to develop beyond 2-cells and 1–2% to morula or blastocyst. Furthermore, the addition of EDTA alone to Medium M16 was ineffective in overcoming the 2-cell block to in-vitro development in these embryos.

Table 2. Culture of 1-cell embryos in various media for 5 days

	Day 4					Day 5						
Sample	Total	%2C	%3–8C	%M	%Abn	Total	%2C	%3–8C	%M	%Bl	%M + Bl	%Abn
M16	50	84	2	0	14	100	44	3	0	0	0	53
WM	75	79	4	0	18	75	37	0	0	0	0	63
EBSS + EDTA*	174	57	0	0	35	160	18	0	0	0	0	76
CZB + glc	100	41	16	18	25	100	8	4	3	8	11	77
CZB	949	4	20	63	13	846	1	4	6	3	9	86

Abbreviations: Total = total no. of embryos tested; 2C = 2-cells; 3–8C = 3–8-cells; M = morula; Bl = blastocysts; Abn = abnormal; glc = glucose.
Experiments were not always scored on both Day 4 and Day 5, therefore totals may be different on the 2 days for a given sample.
*8% of embryos cultured in EBSS and EDTA remained as 1-cells on Day 4 and 6% were still 1-cells on Day 5.

These preliminary results, together with similar published findings for the hamster (Bavister *et al.*, 1983; Carney & Bavister, 1987; Schini & Bavister, 1988; Seshagiri & Bavister, 1989), resulted in the development of the medium designated CZB (Table 1). Compared with Medium M16 or modified BMOC-2 media, CZB medium has a higher lactate/pyruvate ratio, which may be crucial for the development of 1-cell embryos through the 2-cell stage (Cross & Brinster, 1973), lacks glucose, contains 0·1 mM-EDTA (Dr Karl Ebert, personal communication) and 1 mM-glutamine.

Effect of different media on embryo culture

Experiments using CZB medium (without glucose) demonstrated that development of 1-cell CF-1 × B6SJLF1/J embryos through the 2-cell stage to the morula stage was significantly improved over other media tested on these embryos (Table 2). The addition of glucose to Medium CZB was inhibitory to development with only 18% developing to the morula stage by Day 4. By Day 5, however, 77% and 86% of embryos cultured in Medium CZB with or without glucose had become abnormal (Table 2).

Effect of washing variations on embryo culture

The successful development of CF-1 × B6SJLF1/J 1-cell embryos through the 2-cell stage in Medium CZB but the inability of this medium to support the transition from morula to blastocyst led us to question whether there was a stage-specific requirement for glucose during embryo culture even though continuous exposure to glucose was inhibitory to development. To define a window for potential glucose enhancement of development, 1-cell embryos cultured in Medium CZB were washed into Medium CZB + glucose, and in some cases back into Medium CZB, at various times during the culture period. As before, Medium CZB for the duration of the culture period allowed

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72% of embryos to develop to the morula stage on Day 4 but only 11% proceeded to the morula or blastocyst stage on Day 5 (Table 3). Washing into Medium CZB + glucose on Day 2, with or without a wash back into Medium CZB on Days 3 or 4, did not increase the percentage of embryos reaching the morula or blastocyst stage on Day 5. However, a wash into Medium CZB + glucose on Day 3 or injection of glucose directly into the Medium CZB culture drop was sufficient to allow 47% and 70%, respectively, of embryos to develop to morula or blastocysts (Day 5). If embryos were washed into Medium CZB + glucose on Day 3 and then washed back into Medium CZB on Day 4, 63% of embryos developed to morula or blastocysts on Day 5. Embryos washed from Medium CZB into Medium CZB + glucose on Day 4 did not form any blastocysts on Day 5. Treatments in which embryos were exposed to glucose on Day 3 did not differ significantly from one another. However, glucose exposure of any kind on Day 3 caused significant improvements in embryo development (by Student's t test) compared with no wash, wash on Day 2 with or without a wash back into Medium CZB on Day 3 or Day 4, or wash on Day 4. Nuclear counts of embryos which developed to the morula stage were significantly reduced in the samples which were washed into glucose on Days 2 ($9{\cdot}0 \pm 1{\cdot}0$, $10{\cdot}2 \pm 3{\cdot}4$) or 4 ($10{\cdot}3 \pm 1{\cdot}7$) compared with Day 3 ($16{\cdot}2 \pm 1{\cdot}1$) ($P < 0{\cdot}05$). Any embryos which developed to the blastocyst stage regardless of day of glucose exposure did not differ significantly from one another in cell number. These results define a relatively narrow window during which glucose is required by these embryos, beginning at the 3–4-cell stage (Day 3, afternoon) and extending for a maximum of 24 h to the early morula stage (Day 4 afternoon). An exposure to glucose at too early (Day 2, 2-cells) or too late (Day 4, early morula) a time was ineffective in enhancing embryo development.

Following the definition of an optimum time for washing into glucose-containing medium, the type of medium which could be utilized for washing was investigated. Embryos were cultured from the 1-cell stage in Medium CZB and at 48 h were either not washed or washed into Medium CZB, Medium CZB + glucose or Medium M16. Both Medium CZB + glucose and Medium M16 were effective at promoting significant development to the blastocyst stage with 49% morula or blastocysts on Day 5 (Table 4) compared to no wash or Medium CZB. The average number of nuclei per embryo for morulae was $16{\cdot}1 \pm 1{\cdot}34$ ($n = 15$) for Medium CZB + glucose and $14{\cdot}5 \pm 1{\cdot}38$ ($n = 6$) for Medium M16 and for blastocysts was $34{\cdot}9 \pm 1{\cdot}61$ ($n = 30$) for Medium CZB + glucose and $35{\cdot}0 \pm 1{\cdot}75$ ($n = 41$) for Medium M16 (not significantly different). Both the unwashed condition and washing into Medium CZB without glucose, although permitting some development to the morula stage on Day 4, were ineffective at promoting the transition from morula to blastocyst on Day 5 (Table 4).

Timing of glutamine effectiveness

Since washing into Medium M16 (no glutamine) at 48 h was as effective in promoting the morula to blastocyst transition as washing into Medium CZB + glucose, the question of when glutamine exerts its effect was investigated. One-cell embryos were cultured for 48 h in Medium CZB with or without glutamine and washed into Medium CZB + glucose with or without glutamine as described in Table 5. Glutamine was beneficial to embryo development during the first 48 h of culture. Embryos cultured in Medium CZB, regardless of the presence or absence of glutamine in the Medium CZB + glucose wash, developed to the morula stage on Day 4 and to the morula or blastocyst stage on Day 5 (Table 5). Of the embryos cultured in the absence of glutamine for the first 48 h, regardless of the presence or absence of glutamine in the wash, fewer reached the morula stage on Day 4 and only approximately 30% developed to the morula or blastocyst stage on Day 5 (Table 5). By analysis of variance, the presence of glutamine in the medium before washing on Day 3 caused a significant positive effect on development while the presence of glutamine in the medium after washing on Day 3 caused no effect on development. Medium CZB cultures with glutamine, washed on Day 3 into Medium CZB + glucose with or without glutamine, were not significantly different from one another by (Student's t test). Likewise, cultures in Medium CZB without

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Table 3. Effect on 1-cell embryo development of washing into CZB with glucose at various times during the culture period

Sample	Day 4					Day 5						
	Total	%2C	%3–8C	%M	%Abn	Total	%2C	%3–8C	%M	%Bl	%M + Bl	%Abn
CZB	72	3	18	72	7	46	0	0	2	9	11*	89
$CZB^{D2} \rightarrow +$ glc	48	10	31	44	15	48	2	15	0	8	8*	75
$CZB^{D2} \rightarrow +$ $glc^{D3} \rightarrow$ CZB	25	0	28	68	4	50	0	10	4	8	12*	78
$CZB^{D2} \rightarrow +$ $glc^{D4} \rightarrow$ CZB	24	0	8	88	4	49	4	2	6	4	10†	84
$CZB^{D3} \rightarrow +$ glc	100	2	19	71	7	100	1	1	12	35	47*†	51
$CZB^{D3} \rightarrow$ inject glc	101	1	14	83	2	101	0	0	13	57	70	30
$CZB^{D3} \rightarrow +$ $glc^{D4} \rightarrow$ CZB	26	4	0	96	0	126	0	0	13	50	63	37
$CZB^{D4} \rightarrow$ glc	51	2	21	65	12	51	0	0	10	0	10*	90

Abbreviations: Total = total no. of embryos tested; 2C = 2-cells; 3–8C = 3–8-cells; M = morulae; Bl = blastocysts; Abn = abnormal; glc = glucose. D2 = Day 2; D3 = Day 3; D4 = Day 4; for example $CZB^{D3} \rightarrow +$ $glc^{D4} \rightarrow$ CZB, embryos were cultured in CZB medium for 48 h, on Day 3 they were washed into Medium CZB + glucose and washed back into Medium CZB on Day 4.
Experiments were not always scored on both Day 4 and Day 5, therefore totals may be different on the 2 days for a given sample.
*Statistically significantly different from $CZB^{D3} \rightarrow +$ glc, by Student's t test, $P \leqslant 0{\cdot}01$.
†Statistically significantly different from $CZB^{D3} \rightarrow +$ glc, by Student's t test, $P \leqslant 0{\cdot}05$.

Table 5. Effect of glutamine on 1-cell embryo development before and after washing into medium with glucose

Sample	Day 4					Day 5					
	Total	%2C	%3–8C	%M	%Abn	%2C	%3–8C	%M	%Bl	%M + Bl	%Abn
$CZB^{D3} \rightarrow$ CZB + glc	101	0	7	91	2	0	3	9	59	68*	29
$CZB^{D3} \rightarrow$ CZB → gln + glc	152	1	6	90	3	0	0	8	57	65*	35
CZB-$gln^{D3} \rightarrow$ CZB + glc	149	6	34	56	4	5	12	4	26	30	53
CZB-$gln^{D3} \rightarrow$ CZB → gln + glc	146	11	38	50	1	5	8	2	31	33	54

Abbreviations: Total = total no. of embryos tested; 2C = 2-cells; 3–8C = 3–8-cells; M = morulae; Bl = blastocysts; Abn = abnormal; glc = glucose; gln = glutamine; D3 = Day 3; for example: $CZB^{D3} \rightarrow$ CZB + glc, embryos were cultured in CZB medium for 48 h, on Day 3 they were washed into Medium CZB + glucose for the remainder of the culture.
*Statistically significant by analysis of variance, $P \leqslant 0{\cdot}001$.

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Table 4. Effect of washing embryos from CZB into various media at 48 h of culture

	Day 4					Day 5						
Sample	Total	%2C	%3–8C	%M	%Abn	Total	%2C	%3–8C	%M	%Bl	%M + Bl	%Abn
No wash	50	6	22	58	14	75	1	1	0	1	1*†	97
CZB	75	9	5	53	32	99	5	1	10	5	15*†	79
CZB + glc	75	3	11	80	7	99	2	2	18	31	49*	46
M16	76	1	12	86	3	102	1	0	6	43	49†	50

Abbreviations: Total = total no. of embryos tested; 2C = 2-cells; 3–8C = 3–8-cells; M = morulae; Bl = blastocysts; Abn = abnormal; glc = glucose.
Experiments were not always scored on both Day 4 and Day 5, therefore totals may be different on the 2 days for a given sample.
*Statistically significantly different from CZB + glc, by Student's t test, $P \leqslant 0{\cdot}001$.
†Statistically significantly different from M16, by Student's t test, $P \leqslant 0{\cdot}01$.

glutamine, regardless of wash, were not significantly different from each other. Embryos which proceeded to the morula or blastocyst stage in any treatment did not differ significantly from each other in total numbers of nuclei.

Effect of glutamine and glucose on embryo culture

To determine the relative effects of glucose and glutamine on development, a 2^2 factorial experiment was performed (Table 6). One-cell embryos were cultured in Medium CZB with or without glucose and/or glutamine for 48 h and were then washed into identical medium containing glucose for the remainder of the culture period. Glucose, although required after Day 3, was detrimental to embryo development during the first 48 h of culture (Table 6). Glutamine enhanced the rate of morula and blastocyst formation on Day 5, both in the presence and absence of glucose. Optimal development occurred in Medium CZB which contained 1 mM-glutamine but no glucose for the first 48 h. By analysis of variance, the removal of glucose and the addition of glutamine both caused independently significant improvements in embryo development. There was no significant interaction detected between glucose removal and glutamine addition by this analysis.

Table 6. Effect of glutamine and glucose on 1-cell embryo development

Sample		Day 4					Day 5					
Gln	Glc	Total	%2C	%3–8C	%M	%Abn	%2C	%3–8C	%M	%Bl	%M + Bl	%Abn
−	+	52	71	21	0	8	34	4	0	0	0	62
+	+	49	18	43	39	0	6	8	2	8	10†	75
−	−	47	11	36	53	0	8	2	2	28	30*	60
+	−	50	0	10	90	0	0	0	6	52	58*†	42

Abbreviations: Total = total no. of embryos tested; 2C = 2-cells; 3–8C = 3–8-cells; M = morulae; Bl = blastocysts; Abn = abnormal; glc = glucose; gln = glutamine. All samples were washed on Day 3 into the same variant but with glucose.
*Removal of glucose was statistically significant by analysis of variance, $P = 0{\cdot}007$.
†Addition of glutamine was statistically significant by analysis of variance, $P = 0{\cdot}05$.

Discussion

In the present study, the 2-cell block to in-vitro development of 1-cell CF-1 × B6SJLF1/J embryos has been overcome by optimizing the culture conditions and the medium used for culture. These embryos were shown to block at the 2-cell stage in Medium M16, WM and EBSS with EDTA.

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Continuous culture of 1-cell embryos in CZB medium which has a lactate/pyruvate ratio of 116, contains 1 mM-glutamine, 0·1 mM-EDTA and lacks glucose resulted in the development of 83% of embryos beyond the 2-cell stage, but only 9% of these embryos formed blastocysts. A protocol that involved culture of 1-cell embryos for 48 h in CZB medium followed by a transfer into Medium CZB + glucose for the remaining 48 h of the culture period led to an average of 58% of all 1-cell embryos developing to the morula or blastocyst stage. These results have been confirmed for 1-cell embryos from CF-1 donors by Dr Richard Schultz and Dr Joanne Conover, University of Pennsylvania (using a wash into WM at 48 h) and Dr John Biggers, Dr Joel Lawitts and Susan Palmieri, Harvard Medical School (using a wash into Medium M16 at 48 h), and for 1-cell embryos from random bred Swiss mice by Dr Robert Petters, Dr Anthony Archibong, Rebecca Krisher and Betsy Houston, North Carolina State University. CZB medium for these experiments was freshly prepared at each location and appears to be reproducible in other laboratories.

The removal of glucose from the medium during the first 48 h of culture led to a significant improvement in embryo development beyond the 1-cell stage. Brinster (1965c) observed a slightly detrimental effect of glucose on development of 2-cell random-bred Swiss mouse embryos. In the hamster, Schini & Bavister (1988) demonstrated that glucose (in the presence of phosphate) contributes to the hamster 2-cell block (only 2·7% beyond 2-cells). Elimination of glucose from their medium resulted in development of 27% of embryos beyond the 2-cell stage. There is also an 8-cell block to in-vitro development in the hamster. Seshagiri & Bavister (1989) have shown that the development of 8-cell embryos to blastocysts was depressed to 50% in the presence of 5·0 mM-glucose compared with 88% in the same medium lacking glucose.

The glucose inhibition of 1-cell mouse embryo development is confined to the first 48 h of culture. Washing embryos before this time into glucose containing medium is detrimental to blastocyst formation. After 48 h in the absence of glucose, exposure of embryos to glucose for a maximum of 24 h promoted development to the blastocyst stage. Continuous exposure beyond 24 h, although not detrimental, is not required for blastocyst formation. Development from the 8-cell to the blastocyst stage in the absence of glucose has been independently observed previously by Brinster (1965c) and more recently by F. Manejwala & R. Schultz (personal communication). After 72 h of culture from the 1-cell stage in the absence of glucose, embryos cannot be rescued by exposure to glucose-containing medium.

In the present study, glutamine promoted significant improvement in development from the 1-cell stage to the blastocyst stage. Its primary influence is exerted during the first 48 h of culture. Glutamine is accumulated by the 1–2-cell mouse embryo (Brinster, 1971) to a greater extent than at least 10 other amino acids. Glutamine has been implicated as an important amino acid in the maturation and development of hamster (Gwatkin & Haidri, 1973) and rabbit (Bae & Foote, 1975) oocytes and in the development of hamster embryos beyond the 2-cell block (Schini & Bavister, 1988) and the 8-cell block (Bavister *et al.*, 1983; Carney & Bavister, 1987). The role of glutamine in the development of the embryo is not clear although in cultured somatic cells glutamine can be utilized as an energy substrate in place of glucose (Eagle *et al.*, 1955; Reitzer *et al.*, 1979). Zielke *et al.* (1978) described a reciprocal regulation of utilization between glucose and glutamine in human diploid fibroblasts. A similar role for glutamine could be postulated for embryos in culture with the presence or absence of glucose regulating the use of glutamine as an energy substrate (Table 6). It is possible that blocking mouse embryos have the ability to metabolize lactate and pyruvate, but do not generate sufficient energy in this way to develop *in vitro*. The addition of glutamine and the removal of glucose may allow increased glutamine metabolism to supply the additional energy substrates required for development of the 1-cell mouse embryo beyond the 2-cell stage. Other mechanisms however, are possible.

These culture experiments were initiated at the Worcester Foundation in the fall of 1987 and at that time, continuous culture in CZB medium without washing into glucose promoted development of 83% of embryos beyond the 2-cell stage with 58% developing to blastocyst. This protocol worked effectively until April of 1988 at which time a wash into glucose at 48 h became essential.

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The reasons for this change in the requirement for glucose are unclear. Experiments are in progress to determine whether this change is due to some seasonal influence on embryos or to uncontrolled variations in culture medium ingredients, particularly the water.

In summary, development *in vitro* beyond the 2-cell stage has been achieved for 1-cell embryos from CF-1 females × B6SJLF1/J males. That these embryos still block in selected media even when mated to an F1 non-blocking strain of males supports the maternal origin of the 2-cell block as described by Muggleton-Harris *et al.* (1982), Goddard & Pratt (1983) and Pratt & Muggleton-Harris (1988). Changes in the medium composition which allowed development beyond the 2-cell stage suggest that glutamine in addition to lactate and pyruvate may be a required energy substrate for in-vitro development of the embryo in some strains of mice, and that glucose is inhibitory during the first two cleavage divisions *in vitro*.

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