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Development of 1-Cell Embryos from Different Strains of Mice in CZB Medium[1]

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ABSTRACT

One-cell embryos from several different strains of mice have been cultured to the blastocyst stage in CZB medium. CZB medium can be used to culture CF1 × B6SJLF1/J 1-cell embryos to the blastocyst stage provided glucose is introduced into the medium on Day 3 of culture. The amount of glucose required for embryo development was titrated using a concentration range of 5.5 to 49.5 mM. With the exception of the highest concentration, all glucose levels tested supported 65 – 85% development to the morula and blastocyst stages. Variations of CZB medium were tested for their ability to support the development of 1-cell embryos from 4 strains of mice. For embryos from CF1 and DBA/2J (both × B6SJLF1/J) mice, which exhibit a "2-cell block" to development in vitro, CZB medium containing glutamine with the addition of glucose on Day 3 supported optimum development from the 1-cell stage to morula and blastocysts (79% and 87%). For embryos from B6D2F1/J and CD1 female mice (both × B6SJLF1/J males), which do not exhibit a "2-cell block" to in vitro development, optimum development to morula and blastocyst stages (95% and 50%) was in CZB medium containing both glutamine and glucose from the start of culture.

INTRODUCTION

Success with culture of 1-cell mouse embryos to the blastocyst stage has been shown to be a function of both strain of mouse and culture conditions. In early studies, investigators succeeded in culturing embryos from F1 hybrid animals but not from most random-bred strains and some inbred strains (Whitten and Biggers, 1968; Kaufman and Sachs, 1976; Goddard and Pratt, 1983). Whitten and Biggers (1968) cultured F1 hybrid embryos (from a C57B1/10J × SJL/J cross) with 75% of embryos developing to the blastocyst stage in a bicarbonate-buffered medium containing lactate, pyruvate, and glucose. In contrast, that study (Whitten and Biggers, 1968) also showed that the inbred strains SJL/J, C57B1/10J, 129/Rr, and a random-bred strain developed to the blastocyst stage at rates of only 3, 18, 17, and 0%, respectively. Biggers (1971) subsequently confirmed that several hybrid strains (B6AF1, C57 × SJL, B6D2F1) and an inbred strain (C3H) were capable of 30 – 60% development to the blastocyst stage in a defined bicarbonate-buffered medium, while other strains (C3H × DBA, C57, DBA, and Swiss mice) were incapable of development beyond the 2-cell stage (0 – 8% blastocysts) in this medium.

A variety of improvements in embryo culture medium have resulted in increased success in the culture of embryos from some strains whose development in vitro is arrested at the 2-cell stage, i.e. the "2-cell block." However, many of these improvements have not worked equally well in other laboratories. It is unclear whether the differences are environmental (H_2O, gas, etc.) or are due to different strains (and/or their suppliers) in use in these laboratories. Cross and Brinster (1973) demonstrated an optimum lactate/pyruvate ratio of 120 for the development of 1-cell Swiss mouse embryos beyond the 2-cell stage. Later, Abramczuk et al. (1977) reported that the addition of ethylenediaminetetraacetic acid (EDTA) to Whitten's medium was beneficial in promoting the development of 1-cell embryos from ICR, C57B1/6, and, to a lesser extent, Balb/c mice to the blastocyst stage. More recently, Loutradis et al. (1987) have shown that hypoxanthine can cause developmental arrest at the 2-cell stage in vitro in CD1 1-cell embryos, although hybrid embryos B6D2F1 do not appear to be affected.

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One-cell embryos from CF1 mice, which do not develop beyond the 2-cell stage in most media, have recently been cultured to the blastocyst stage in CZB medium (Chatot et al., 1989). This medium is a modification of BMOC-2 (Ebert and Brinster, 1983) containing an increased lactate/pyruvate ratio (116), 0.1 mM EDTA, 1 mM glutamine and lacking glucose. (BMOC-2 medium caused a 2-cell block for 1-cell CF1 × B6SJLF1/J embryos when tested in our laboratory; unpublished data, Ziomek et al.) Chatot et al. (1989) showed that culture in CZB medium, when supplemented with glucose at 48 h of culture, allowed development of 83% of embryos beyond the 2-cell stage, with 58% of them developing to the morula or blastocyst stage. The CF1 strain of females was chosen in that initial study because embryos from it block at the 2-cell stage but also because CF1 is a cost-effective strain both in terms of animal cost and increased embryo yields in response to superovulation (see *Materials and Methods* section). The preparation and successful use of this medium for development of blocking strains of mice (CF1 and Swiss Webster) beyond the 2-cell stage was reproducible in three different laboratories (Chatot et al., 1989), suggesting that it may be superior to other media for the culture of 1-cell embryos from blocking strains of mice. In support of this for CF1 embryos cultured from the 1-cell stage, Poueymirou et al. (1989) recently have demonstrated that total protein synthetic rates at the 2-cell stage and the onset and rate of protein synthesis derived from embryonic genome transcripts are enhanced in CZB medium when compared with Whitten's medium with or without EDTA.

The present study extends these previous results to determine 1) if there is an optimum concentration of glucose required at 48 h of culture to promote development of CF1 × B6SJLF1/J embryos from the morula to the blastocyst stage and 2) if this medium can be used to culture 1-cell embryos from other strains of mice, one that exhibits a block at the 2-cell stage and two that do not. We have tested CZB medium for its effectiveness in promoting the development of 1-cell embryos beyond the 2-cell stage to the blastocyst stage using embryos from CF1, DBA/2J, B6D2F1/J, and CD1 females, all mated with B6SJLF1/J males.

MATERIALS AND METHODS

Embryo Collection

Female mice used in this study were of the blocking strains CF1 (Harlan Sprague-Dawley, Colony 202, Indianapolis, IN) and DBA/2J (Jackson Laboratories, Bar Harbor, ME) and the nonblocking strains B6D2F1/J (an F1 hybrid from C57B1/6J × DBA/2J, Jackson Laboratories) and CD1 (Charles River Breeding Laboratories, Wilmington, MA). Mice were superovulated with i.p. injections of 10 IU pregnant mare's serum gonadotropin (PMSG, Calbiochem, La Jolla, CA,) followed 48 h later by 5 IU human chorionic gonadotropin (hCG, Organon, W. Orange, NJ). CF1, DBA/2J, B6D2F1/J, and CD1 females were mated overnight with B6SJLF1/J males (an F1 hybrid from C57B1/6J × SJL/J, Jackson Laboratories). Embryos were flushed from excised oviducts at 25 – 27 h after hCG (Day 1 of culture) into Hanks' balanced salt solution with 4 mg bovine serum albumin/ml (HBSS+BSA), treated briefly if necessary with hyaluronidase (300 units/ml in phosphate-buffered saline [PBS] containing 1% polyvinylpyrrolidone, Sigma Chemical Co., St. Louis, MO) to remove cumulus cells, washed 3 times in HBSS+BSA, and placed in holding drops of CZB medium as previously described (Chatot et al., 1989). Average yields of 1-cell embryos per animal were 55.1 (n=28 mice) for CF1 × B6SJLF1/J, 18.3 (n=41) for DBA × B6SJLF1/J, 26.1 (n=33) for B6D2F1/J × B6SJLF1/J, and 12.6 (n=50) for CD1 × B6SJLF1/J. Embryos from each mouse were kept in separate 50-μl holding drops of CZB medium until adequate numbers of embryos were collected and then were randomly distributed across all samples in each experiment. All manipulations were performed in a darkened room (Schumacher and Fischer, 1988) and all solutions, dishes, and instruments were maintained at 37°C prior to use.

Culture Media

Embryos were cultured in CZB medium (for formula, see Chatot et al., 1989), which contains 1 mM glutamine, 0.1 mM EDTA, and 5 mg/ml BSA and lacks glucose unless otherwise indicated. CZB medium was prepared on the basis of weight using sterile endotoxin-free tissue culture grade water (Cat. #W3500, Sigma Chemical Co.) as described (Chatot et al., 1989). When glutamine was present in the medium, it was added immediately prior to use from a freshly prepared 100 mM stock to give a final concentration of 1 mM. When glucose was present in the medium from the start of culture, it was added to the medium to give a final concentration of 5.56 mM (1 mg/ml). Medium was stored gassed with 5% CO_2:5% O_2:90% N_2 at 4°C and was freshly prepared every 2 wk.

Culture Procedures

Media were pipetted in 50-μl drops into Falcon 35-mm petri dishes (Falcon Cat. #1008, Beckton-Dickinson, Lincoln Park, NJ) overlaid with CZB medium-washed Fisher 121 paraffin oil, gassed in a sealed culture chamber (Billips Rothenberg, Del Mar, CA) with 5% CO_2:5% O_2:90% N_2 and equilibrated at 37°C for 4 – 5 h prior to use (Chatot et al., 1989). Randomly distributed 1-cell embryos were removed from the holding dishes, washed through a drop of fresh medium, and placed into a final culture drop or dish. A maximum of 25 embryos were cultured in a 50-μl drop. Cultures were placed in a sealed culture chamber, gassed as described above, and incubated at 37°C for 96 h (Day 5 of culture). Cultures that required an exposure to glucose were removed from the culture chamber at 48 h of culture (Day 3); 2.5 μl (unless otherwise indicated) of a sterile 100-mg glucose/ml dH_2O stock was injected directly into the culture drop, and cultures were returned to the culture chamber for continued incubation. In some cases, embryos were washed from their original drop of CZB culture medium into a fresh drop of CZB medium containing glucose at the indicated concentration. Embryos were scored for developmental stage and abnormalities at both 72 (Day 4) and 96 h (Day 5) of culture and cultures were terminated at 96 h (Day 5).

Nuclear Counts

Embryos grown in vitro were fixed at 96 h of culture in 4% paraformaldehyde, washed into phosphate-buffered saline (PBS), and stained with Hoechst 33258 (10 μg/ml in HBSS+BSA, Cat. #B2883, Sigma Chemical Co.) to label the nuclei fluorescently. For nuclear counts on in vivo blastocysts, embryos were flushed from uteri of CF1, DBA/2J, B6D2F1/J, and CD1 females 96 h after hCG and mating with B6SJLF1/J males (Day 4 of pregnancy). Day 4 (rather than Day 5) in vivo blastocysts were utilized for a comparison with cultured embryos, because cultured embryos tend to develop more slowly than in vivo embryos and, for many strains, it is difficult to isolate intact embryos from the uterus on Day 5 because implantation has already begun. Embryos were fixed and stained as described for cultured embryos. Nuclei were counted on a Nikon Diaphot inverted phase-contrast microscope with fluorescence attachments under U.V. illumination. Average nuclei per morula and per blastocyst were calculated.

Statistical Analysis

To control for variation between litters, embryos from each animal were randomly distributed across samples in a given experiment. Each experimental protocol represents between 3 and 8 repetitions performed on different days and by different individuals. For all experiments, a complete set of samples was run simultaneously, e.g. for experiments with different strains, all six samples were run for a given strain at one time. As a control for seasonal or environmental variations, a CF1 matrix was run with every experiment, because this blocking strain is the best-characterized standard for development in CZB medium. Statistical analysis of experiments was performed with an NCSS software package (Number Crunching Statistical System, NCSS, Kayville, UT) using a standard arcsin transformation (Sokal and Rohlf, 1981) of the data and analysis of variance or Student's *t*-test. For analysis of variance, the six experimental samples described in each strain experiment (Tables 2 – 5) were divided into two 2^2 factorial matrices: 1) four samples all without a Day 3 glucose injection and with or without glutamine (gln) and glucose (glc) as described, i.e. – gln – glc, – gln+glc, +gln – glc, +gln+glc; and 2) four samples with or without a Day 3 glucose injection and with or without glutamine only, i.e. – gln – glc, +gln – glc, – gln+glc Day 3, +gln+glc Day 3. Analysis of morula and blastocyst cell numbers was performed on non-transformed nuclear counts using Student's *t*-test.

RESULTS

Titration of Day 3 Glucose Injection Concentration

In a previous study (Chatot et al., 1989), it was determined that CF1 embryos could be cultured from the 1-cell stage to the blastocyst stage in CZB medium, but required an exposure to glucose at 48 h of culture to develop successfully through the morula to blastocyst transition. In that study, embryos were washed at 48 h into a fresh drop of CZB medium containing 1 mg glucose/ml (5.56 mM); however, it was noted that injection of 2.5 μl of a 100-mg glucose/ml stock (giving a final concentration of 27.5 mM) into the original culture drop was equally successful. To determine if this high glucose concentration in the injection protocol was detrimental to embryos in culture, CF1 × B6SJLF1/J 1-cell embryos were cultured in CZB medium, and the concentration of glucose introduced into the culture

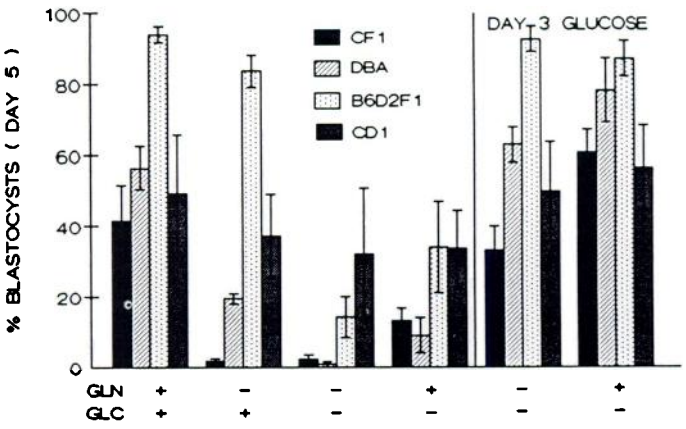

FIG. 1. Summary of percent blastocyst development on Day 5 from 1-cell embryos from different strains of mice in variations of CZB medium. For all strains listed, embryos were isolated from females after mating with B6SJLF1/J males. Abbreviations: GLN, glutamine; GLC, glucose. Data are represented ± SEM as indicated by *error bars*.

at 48 h was titrated, both by direct injection into the culture drop (5.5 – 49.5 mM) or by washing the embryos into a fresh drop of medium containing glucose (5.56 – 27.8 mM) (Table 1). In the absence of glucose injection when washed into glucose-free CZB medium (Table 1), embryos developed normally, by morphological criteria, to the morula stage but subsequently deteriorated to abnormal between Days 4 (72 h) and 5 (96 h) as previously determined (Chatot et al., 1989). Regardless of glucose concentration or method of glucose exposure, 90 – 99% of embryos developed beyond the 2-cell stage and, with the exception of the 49.5 mM glucose concentration, 48 – 85% of embryos developed to the blastocyst stage. At the highest glucose concentration used, embryo development was generally slowed at the 3-cell to morula stages. However, there were no statistically significant differences between any of the treatments by Student's *t*-test on Day 4 (morula and blastocyst) or Day 5 (blastocyst). Therefore, in subsequent experiments, exposure to glucose at 48 h of culture on Day 3 was by direct injection of 2.5 µl of 100 mg glucose/ml stock (27.5 mM) into microdrop cultures.

Culture of 1-Cell Mouse Embryos from Different Strains in Variations of CZB Medium

To determine if CZB medium was optimal for in vitro development of 1-cell embryos from a variety of different strains, the following matrices were performed. Embryos were cultured 1) in CZB medium with and without 1 mM glutamine and 5.56 mM glucose for the entire culture period without a Day 3 exposure to glucose and 2) in CZB medium with and without glutamine and with and without a Day 3 exposure to glucose as indicated in Tables 2 – 5 and Figure 1.

CF1 × B6SJLF1/J embryos. For CF1 × B6SJLF1/J 1-cell embryos, in the no Day 3 glucose injection condition, the presence of both glutamine and glucose supported development to the blastocyst stage (Table 2). Both the presence of glutamine and the presence of glucose for the first type of matrix were statistically significant by analysis of variance and there was a significant interaction detected between them (Table 6). However, CF1 × B6SJLF1/J 1-cell embryos required both glutamine and a Day 3 exposure to glucose for

TABLE 1. Titration of glucose concentration by injection and wash regimes at 48 h of CF1 × B6SJLF1/J embryo culture.*

		Day 4						Day 5						
Sample	N	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	% M+Bl
Injection														
No injection	7	162	7	6	10	77	0	164	60	5	2	17	15	32
0.5 µl = 5.5 mM	3	75	1	0	7	88	4	75	24	0	0	12	64	76
1.5 µl = 16.5 mM	3	75	0	1	4	92	3	75	16	1	0	16	67	83
2.5 µl = 27.5 mM	3	72	1	0	7	89	3	72	18	1	3	14	64	78
3.5 µl = 38.5 mM	3	74	1	1	3	93	1	75	15	0	0	0	85	85
4.5 µl = 49.5 mM	3	75	3	7	29	60	3	75	29	7	13	9	41	51
Wash														
CZB no glucose	7	174	19	4	3	73	1	175	58	3	0	10	29	39
1 mg/ml = 5.5 mM	3	75	3	0	5	88	4	74	23	0	0	15	62	77
2 mg/ml = 11.1 mM	3	75	0	1	7	92	0	75	27	1	1	20	51	77
5 mg/ml = 27.8 mM	3	75	1	0	8	88	3	75	31	1	1	17	48	65

*Abbreviations: N, number of replicates; Total, total number of embryos; Abn, abnormal; 2C, 2-cell; 3 – 8C, 3- to 8-cell; M, morula; Bl, blastocyst.

TABLE 2. Culture of CF1 × B6SJLF1/J 1-cell embryos in variations of CZB medium.*

			Day 4						Day 5						
Gln	Glc	N	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	% M+Bl
No Day 3 glucose															
–	–	8	186	7	5	13	75	1	186	83	1	4	8	4	12
–	+	8	186	13	39	38	10	0	184	47	30	17	3	3	6
+	–	8	187	6	6	8	80	0	187	64	5	2	14	15	29
+	+	8	190	6	5	19	66	3	189	31	8	6	10	46	56
Day 3 glucose injection															
–	–	8	189	5	6	14	75	0	189	41	5	7	12	35	47
+	–	8	189	4	1	4	88	3	189	19	1	2	15	64	79

*Abbreviations: N, number of replicates; Total, total number of embryos; Abn, abnormal; 2C, 2-cell; 3 – 8C, 3- to 8-cell; M, morula; Bl, blastocyst; Gln, glutamine; Glc, glucose.

optimal development to the blastocyst stage, with 64% blastocysts compared to 3 – 46% for any other treatment (Table 2 and Figure 1). By analysis of variance of the second type of matrix, i.e. with and without glutamine and with and without a Day 3 glucose injection, the presence of glutamine (p=0.0008) and the Day 3 glucose injection (Table 6) contributed significantly to embryo development, although there was no interaction between them. This confirmed our previous results (Chatot et al., 1989) that CZB medium with a Day 3 injection of glucose was optimum for development of CF1 × B6SJLF1/J 1-cell embryos to blastocyst. In CZB medium with a Day 3 glucose injection, average blastocyst cell number was 30.70 ± 0.91 (n=115). However, any blastocysts that formed in the presence of glucose (either from Day 1 or Day 3) did not differ significantly in total cell number. In the absence of any glucose during the culture period, blastocysts had significantly fewer cells, 20.65 ± 1.53 (n=20) ($p \leq 0.001$) (for +gln – glc) and 22.50 ± 3.91 (n=8) ($p \leq 0.03$) (for – gln – glc). CF1 × B6SJLF1/J blastocysts isolated on Day 4 of gestation averaged 21.43 ± 0.35 (n=240) cells and had significantly fewer cells than those cultured for 96 h in CZB medium with a Day 3 glucose injection (p=0.0142 by Student's t-test).

DBA/2J × B6SJLF1/J embryos. Embryos from another strain that arrests development in vitro at the 2-cell stage in some media were also cultured in these variations of CZB medium to assess development to the blastocyst stage. DBA/2J × B6SJLF1/J 1-cell embryos cultured in CZB medium in the absence of a Day 3 glucose injection developed beyond the 2-cell stage in all media tested. However, on Day 4 in the absence of glutamine, development to the morula or blastocyst stage was reduced, with embryos either slowing development at the 3- to 8-cell stage or becoming abnormal (Table 3). On Day 5, development to the blastocyst stage was significantly improved over other variations in the first type of matrix when both glutamine and glucose were present in the medium (Tables 3 and 6 and Figure 1), and there was a significant interaction between these two components detected by analysis of variance (Table 6). Like 1-cell embryos from CF1 × B6SJLF1/J matings, DBA/2J × B6SJLF1/J 1-cell embryos developed optimally to the blastocyst stage in CZB medium containing glutamine from Day 1 with a

TABLE 3. Culture of DBA/2J × B6SJLF1/J 1-cell embryos in variations of CZB medium.*

			Day 4						Day 5						
Gln	Glc	N	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	% M+Bl
No Day 3 glucose															
–	–	5	111	34	1	3	62	0	111	94	1	4	1	1	2
–	+	5	112	28	5	24	43	1	112	66	3	6	4	20	24
+	–	5	110	3	1	2	94	0	110	88	0	2	0	10	10
+	+	5	109	3	3	8	72	15	109	28	1	2	13	57	70
Day 3 glucose injection															
–	–	5	110	7	2	4	78	8	110	24	1	2	11	63	74
+	–	5	103	1	2	4	69	24	103	9	2	3	10	77	87

*Abbreviations: N, number of replicates; Total, total number of embryos; Abn, abnormal; 2C, 2-cell; 3 – 8C, 3- to 8-cell; M, morula; Bl, blastocyst; Gln, glutamine; Glc, glucose.

TABLE 4. Culture of B6D2F1/J × B6SJLF1/J 1-cell embryos in variations of CZB medium.*

			Day 4						Day 5						
Gln	Glc	N	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	% M+Bl
No Day 3 glucose															
–	–	5	123	51	0	0	34	15	123	85	1	0	0	14	14
–	+	5	118	4	0	3	21	72	118	14	0	0	2	84	86
+	–	5	121	38	0	1	36	25	122	63	0	0	3	34	37
+	+	5	122	5	0	0	3	94	121	5	0	0	0	95	95
Day 3 glucose injection															
–	–	5	123	4	0	0	39	57	123	6	0	0	2	93	94
+	–	5	123	4	1	0	27	68	123	12	0	0	1	87	88

*Abbreviations: N, number of replicates; Total, total number of embryos; Abn, abnormal; 2C, 2-cell; 3 – 8C, 3- to 8-cell; M, morula; Bl, blastocyst; Gln, glutamine; Glc, glucose.

Day 3 glucose injection. In this medium, 77% of embryos developed to the blastocyst stage by Day 5. The presence of glutamine and the Day 3 glucose injection in the second type of matrix both contributed significantly to this effect (p=0.048 and p<0.0001, respectively, by analysis of variance). Blastocysts that developed in any CZB media variant containing glucose were not significantly different from one another and ranged in cell number from 31.67 ± 3.15 (n=15) (in – gln + glc) to 38.41 ± 1.99 (n=64) (in – gln Day 3 glc). Blastocysts cultured in CZB medium with glutamine and a Day 3 glucose injection averaged 35.48 ± 1.42 cells (n=75). This is significantly larger than blastocysts isolated from in vivo on Day 4 of gestation that averaged 28.57 ± 0.96 cells (n=104) (p=0.0001 by Student's t-test). It is also interesting to note that on Day 4, only 41% (104/252) of embryos isolated from in vivo were blastocysts, with the remaining 59% of embryos (148/252) being morulae with an average cell number of 18.13 ± 0.50. In vitro on Day 4, embryos cultured in CZB medium with glutamine and with a Day 3 glucose injection were 69% morula and 24% blastocysts.

B6D2F1/J × B6SJLF1/J embryos. One-cell embryos from two strains of mice that are not generally reported to arrest development in vitro at the 2-cell stage were also evaluated for their ability to develop in vitro in variations of CZB medium. For 1-cell embryos from the cross B6D2F1/J × B6SJLF1/J, like the other strains described, glutamine in the absence of a Day 3 glucose exposure was significantly beneficial (Tables 4 and 6 and Figure 1). However, unlike the blocking strains for which glucose is inhibitory when added at Day 1 compared to injection on Day 3 of culture, these 1-cell embryos developed well in all variations of CZB medium that contained glucose. Based on total cell numbers on Day 5 as well as percent development to the blastocyst stage on Days 4 and 5, optimal development of B6D2F1/J × B6SJLF1/J 1-cell embryos occurred in CZB medium with glutamine and glucose present from Day 1. Average blastocyst cell number under this condition was 58.25 ± 2.42 (n=110). Blastocysts isolated from in vivo on Day 4 averaged only 36.89 ± 0.83 cells (n=284), which is significantly different from the optimum in vitro condition (p<0.0001 by Student's t-test).

CD1 × B6SJLF1/J embryos. One-cell CD1 × B6SJLF1/J embryos developed to the blastocyst stage to an equivalent extent in all variations of CZB medium

TABLE 5. Culture of CD1 × B6SJLF1/J 1-cell embryos in variations of CZB medium.*

			Day 4						Day 5						
Gln	Glc	N	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	Total	% Abn	% 2C	% 3 – 8C	% M	% Bl	% M+Bl
No Day 3 glucose															
–	–	5	121	31	16	6	31	17	121	56	8	5	1	30	31
–	+	5	120	8	18	15	47	13	117	45	10	4	4	37	41
+	–	5	131	22	14	1	46	11	131	50	11	4	3	31	34
+	+	5	117	13	18	13	25	32	117	34	12	4	3	47	50
Day 3 glucose injection															
–	–	5	121	9	14	10	53	14	120	33	11	2	6	48	54
+	–	5	132	7	11	6	60	17	131	31	8	4	3	53	56

*Abbreviations: N, number of replicates; Total, total number of embryos; Abn, abnormal; 2C, 2-cell; 3 – 8C, 3- to 8-cell; M, morula; Bl, blastocyst; Gln, glutamine; Glc, glucose.

TABLE 6. Strain matrices analysis of variance summary.*

	Day 4				Day 5			
Strain	Gln[a]	Glc[a]	Gln × Glc[a]	+/–D3 Glc[b]	Gln[a]	Glc[a]	Gln × Glc[a]	+/–D3 Glc[b]
CF1	0.0002	0.0000	0.0197	NS	0.0002	0.0162	0.0131	0.0000
DBA	0.0037	NS	NS	0.0004	0.0001	0.0000	0.0029	0.0000
B6D2F1	NS	0.0009	NS	NS	0.0344	0.0000	NS	0.0000
CD1	NS	NS	NS	NS	NS	NS	NS	NS

*Abbreviations: Gln, glutamine; Glc, glucose; Gln × Glc, represents interaction of glutamine and glucose; +/–D3 Glc, with and without a Day 3 glucose injection.

[a]Analysis of variance for matrix 1; samples are with and without glutamine and with and without glucose from Day 1 of culture only.

[b]Analysis of variance for matrix 2; samples are with and without glutamine and with and without a Day 3 glucose injection.

tested. Neither the presence of glutamine nor the presence of glucose made any significant improvement in the ability of these embryos to develop to blastocysts (Tables 5 and 6 and Figure 1). The percent blastocysts on Day 5 ranged from 30% in the absence of both glutamine and glucose to 53% in the presence of glutamine with a Day 3 glucose injection. Based on average cell numbers, however, CZB with glutamine and glucose from Day 1 gave significantly more cells per blastocyst, 46.06 ± 2.65 cells (n=52), than CZB with glutamine and a Day 3 glucose injection, 36.52 ± 2.04 (n=60) (p=0.005 by Student's t-test). Blastocysts isolated from in vivo on Day 4 averaged 29.75 ± 0.74 cells (n=71) (p<0.001 compared to CZB with glutamine and glucose from Day 1, by Student's t-test).

DISCUSSION

CZB medium has been evaluated for its ability to support the growth and development of 1-cell embryos from a number of different strains of mice. One-cell embryos from two strains of mice that reportedly exhibit a "2-cell block" to development in vitro (CF1 × B6SJLF1/J and DBA/2J × B6SJLF1/J) both demonstrated a requirement for glutamine and a Day 3 glucose injection to develop optimally to the blastocyst stage. Chatot et al. (1989) have shown for CF1 × B6SJLF1/J embryos that a requirement for glucose arises at the 4- to 8-cell stage and is limited in time to 24 h, since embryos could be washed into glucose-free medium at this time without detrimental effects on development to the blastocyst stage. The reason for this requirement is unclear, although this period parallels the time at which the embryo begins active glucose uptake and metabolism (Brinster and Thomson, 1966; Wales and Brinster, 1968; Gardner and Leese, 1988). Although the embryo can utilize glucose at this stage, it may not absolutely require it, as Brinster (1965) reported the development of 8-cell stage embryos to the blastocyst stage in the absence of glucose.

Glucose present from Day 1, although contributing to development, was less beneficial for CF1 and DBA (both × B6SJLF1/J) compared to the addition of glucose to the cultures on Day 3. Brinster (1965) reported a slightly detrimental effect of glucose on 2-cell random-bred Swiss mice. In hamsters, glucose in the presence of phosphate allows only 2.7% development beyond the 2-cell stage, whereas in the absence of glucose, 27% of embryos develop beyond 2-cells (Schini and Bavister, 1988). A similar effect has been demonstrated for the hamster 8-cell block (Seshagiri and Bavister, 1989). That embryos tend to block at the 2-cell stage in the absence of glutamine and the presence of glucose, particularly for CF1 × B6SJLF1/J embryos, supports the maternal origins of the "2-cell block" (localized to the cytoplasm) as suggested by Muggleton-Harris et al. (1982), Goddard and Pratt (1983), and Pratt and Muggleton-Harris (1988), as these female mice have been mated to a nonblocking strain of males. However, some data exist for a role of the nucleus in the "2-cell block" as well (Robl et al., 1988).

One-cell embryos from 2 strains of mice that do not generally exhibit a "2-cell block" demonstrated slightly different requirements. One-cell embryos from B6D2F1/J × B6SJLF1/J and CD1 × B6SJLF1/J matings developed optimally to the blastocyst stage in the presence of both glutamine and glucose for the entire culture period. This suggests an intrinsic difference in the ability of blocking and nonblocking strains of mouse embryos to metabolize glucose. Since all female strains were mated to the same strain of male, this may be indicative of a maternal origin for this difference.

Embryos cultured under optimal conditions for 5 days were compared to embryos isolated from in vivo on Day 4 for the number of cells per morula and per blastocyst. Compared to embryos isolated from in vivo

at Day 4 of gestation, cultured embryos from CF1 and CD1 (× B6SJLF1/J) contained more cells on Day 5, and the majority of embryos did not become blastocysts until late Day 4 or early Day 5. This suggests that these embryos may be half a day behind their in vivo counterparts. However, for DBA and B6D2F1/J (× B6SJLF1/J) embryos, Day 5 blastocysts contained significantly more cells than Day 4 in vivo and the percent morula and blastocyst for these strains on Day 4 in vitro was equivalent to that observed in vivo on Day 4, suggesting that these cultured embryos may be comparable in cell number to in vivo. Additional experiments involving embryo transfer will provide further information on this question.

The addition of glutamine to the medium was an important factor in the development of all of the strains tested. This was previously demonstrated for 1-cell CF1 × B6SJLF1/J embryos (Chatot et al., 1989). In that study, it was shown that glutamine was beneficial to embryos during the first 48 h of culture and that its removal after 48 h was not detrimental to development to blastocyst. Glutamine is accumulated by embryos both in vivo and in vitro at all stages of development (Brinster, 1971; Chatot and Ziomek, 1989) and is utilized by the embryo in part through the trichloracetic acid cycle (Chatot et al., 1990). Glutamine has been implicated as an essential amino acid for the maturation of rabbit (Bae and Foote, 1975) and hamster (Gwatkin and Haidri, 1973) oocytes and is important for the in vitro development of hamster 2-cell (Schini and Bavister, 1988) and 8-cell (Bavister et al., 1983; Carney and Bavister, 1987) embryos beyond their respective culture blocks. Glutamine is used by cells in culture as an energy substrate in place of glucose (Eagle et al., 1955; Reitzer et al., 1979) and probably functions, at least in part, in the same way in the embryo. This additional energy may be enough to allow the embryos to develop through a critical period when lactate and pyruvate do not supply sufficient energy. Glutamine is also an important amino acid in the synthesis of purine and pyrimidine nucleotides (Salzman et al., 1958; Nomura and Rubin, 1988) and, at a time when the embryo is beginning to divide more rapidly, may be required to enhance the nucleotide pool. Glutamine can affect cells in many ways, so other explanations for the embryo requirement are possible.

Strain differences in culture medium requirements for development to the blastocyst stage are reported in the literature. Dandekar and Glass (1987) have shown that 1-cell and 2-cell embryos from Dub:(ICR), CFW, and CF1 embryos respond to varying degrees to Spindle's, Earle's, Ham's F10, T6, and Hoppe and Pitt's media supplemented with 15% human pre-ovulatory serum. In that study, embryo response was both strain-specific and medium-specific, with 1-cell CF1 embryos developing optimally in T6 medium with 15% human preovulatory serum (86% blastocysts). Biggers (1971) reports the inability to culture DBA 1-cell embryos (as well as C3H × DBA, C57, and Swiss) beyond the 2-cell stage (0% blastocysts for DBA) in a defined bicarbonate-buffered medium. Both B6D2F1/J and CD1 embryos have been used extensively in culture experiments. B6D2F1/J 1-cell embryos have been reported to develop to the blastocyst stage in defined bicarbonate-buffered medium (29% blastocysts, Biggers, 1971), BWW medium (63% blastocysts, Loutradis et al., 1987), Modified Whittens (91% blastocysts, Robl et al., 1988), and EBSS+BSA±EDTA (99% and 78% blastocysts respectively, Jackson and Kiessling, 1989). CD1 1-cell embryos have been reported to develop to the blastocyst stage in BWW medium, (39% blastocysts, Loutradis et al. 1987), in Modified Brinster's medium with HEPES buffer (59 – 86% blastocysts, Papaioannou and Ebert, 1986), and EBSS+BSA±EDTA (51% and 18% blastocysts, respectively, Jackson and Kiessling, 1989). Ackerman et al. (1983) demonstrated that in vitro fertilization and subsequent development to the 4-cell stage differed by strain (CD-1, CB6F1, or B6CBAF1) and medium used (modified Kreb's-Ringer bicarbonate medium or Ham's F10). CZB medium used in the present study supported the development of all strains tested, with only slight variations in the requirement for glucose supplementation between "blocking" and "nonblocking" strains. This simple defined medium supported superior development through the 2-cell block to the blastocyst stage for both CF1 and DBA/2J (both × B6SJLF1/J) 1-cell embryos. The simple addition of glucose to this medium at the start of culture supported development of B6D2F1/J and CD1 (both × B6SJLF1/J) to the blastocyst stage at levels at least equivalent to those reported in the literature. The convenience of being able to culture 1-cell embryos from both blocking and nonblocking strains of mouse in one medium (with the simple addition or removal of glucose) allows the use of cost-effective random-bred animals (i.e. CF1) for routine experiments as well as the use of inbred strains (i.e. DBA) for experiments involving genetic analysis.

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