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CSF-1 and mouse preimplantation development in vitro

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SUMMARY

The effects of macrophage colony stimulating factor on the development of the zygote to the blastocyst stage of an outbred strain of mouse have been studied in KSOM, an improved medium that supports a high rate of in vitro development. Macrophage colony stimulating factor accelerates the formation of the blastocyst cavity by day 4 (96 hours post-hCG). It also increases overall embryonic cell number through a differential increase in the number of trophoblast cells, with no significant effect on the number of inner cell mass cells. By day 5 of culture (120 hours post-hCG), colony stimulating factor-treated embryos have about 20 more trophoblast cells than control embryos, an increase of about 30 percent of the total number of cells in a control blastocyst. The maximum response of embryos was obtained at a concentration around 540 U ml^{-1} colony stimulating factor (≡918 Stanley units ml^{-1}), and the cytokine can produce the same effects even if it is present in the medium for only part of the culture period. This in vitro stimulation of preimplantation development with macrophage colony stimulating factor is compatible with continued normal fetal development in vivo.

Key words: macrophage colony-stimulating factor, CSF-1, blastocyst, trophoblast cell, preimplantation development, embryo culture

INTRODUCTION

Several receptors that recognize cytokines, which include growth factors, are expressed in mammalian preimplantation embryos (Schultz and Heyner, 1993). One of these is the receptor for macrophage colony stimulating factor (CSF-1), the product of the c-*fms* protooncogene. *c-fms* mRNA, which is present in the unfertilized mouse oocyte, decreases to undetectable levels during the 1- and 2-cell stage; it reappears at the late 2-cell stage and is present throughout the rest of preimplantation development (Arceci et al., 1992). There is evidence that the receptor is functional during this period of development since the inclusion of CSF-1 in medium increases the yield of blastocysts in embryos cultured from the 2-cell stage (Pampfer et al., 1991). Gene transcripts for the growth factor, CSF-1, are not detected in preimplantation embryos, but both messenger RNA and protein are present in cumulus cells, in the oviduct and in the glandular and luminal uterine epithelium throughout the preimplantation period of pregnancy (Bartocci et al., 1989; Pampfer et al., 1991; Arceci et al., 1992). Although CSF-1-deficient embryos (*op/op*) can develop normally, the infertility of *op/op* × *op/op* matings, and the lower implantation rate and higher resorption rate in *op/op* females mated to *+/op* or +/+ males, clearly indicate a role for CSF-1 in both pre- and post-implantation embryos, although it cannot be considered a primary growth factor (Pollard et al., 1991).

Whitten and Biggers (1968) showed that zygotes produced by the fertilization of ova from F_1 hybrid female mice can develop into blastocysts in a cytokine-free medium. A similar result was later obtained using three inbred lines (Whitten, 1971) and an outbred line (Abramczuk et al., 1977). These results show that exogenous cytokines are not necessary at any stage of preimplantation development for the formation of the blastocyst. In experiments reported by Pampfer et al. (1991), however, the cytokine CSF-1 increased the yield of blastocysts from 54 to 94 percent after four days in culture by accelerating the development of slowly developing embryos. It is unclear whether this effect is due to the mitogenic action of CSF-1 or other actions that cytokines can produce (Arai et al., 1990). We have probed this phenomenon further by determining the concentration-response lines observed when zygotes are exposed to different concentrations of CSF-1, in an improved cytokine-free medium (KSOM) which supports a high rate of development (>90%) of outbred mouse zygotes into blastocysts (Lawitts and Biggers, 1993; Erbach et al., 1994). Several parameters of development and differentiation were used. The time of first appearance of a blastocoele was taken as a measure of stage- and tissue-specific function, specifically the transport function of the blastocyst. Cell numbers in the trophoblast (TB) and inner cell mass (ICM) of blastocysts were determined using a differential staining technique, as a measure of overall mitotic activity and cellular differentiation into the first two cell lineages of the embryo,

MATERIALS AND METHODS

Animals

Outbred female mice (Crl:CF-1 BR, 6-8 weeks old, Charles River) and

hybrid male mice (B6D2F1/Cr1BR, Charles River) were maintained in 14 hours light and 10 hours dark (lights on at 05:00 hours). Female mice were superovulated by intraperitoneal (i.p.) injection of 5 IU pregnant mares' serum gonadotrophin (PMSG; Sigma Chemical Company, St. Louis) at 15.30 hours, followed by an i.p. injection of human chorionic gonadotropin (hCG, Sigma) 48 hours later. Following the hCG injection, females were placed in cages with males and examined the following morning (day 1) for the presence of vaginal plugs.

Chemicals and CSF-1

All chemicals, except CSF-1, were purchased from Sigma Chemical Company. Recombinant CSF-1 (human sequence, carrier-free, 216-MC/CF) was obtained from R & D Systems, Inc., Minneapolis. This preparation consists of the N-terminal amino acid residue necessary and sufficient to interact with the CSF-1 receptor (Wong et al., 1987), which is highly conserved between the human and murine proteins. The biological activity of the recombinant CSF-1 was evaluated by the supplier in a cell proliferation assay. The mean ED-50 was estimated to be 0.35 ng ml^{-1}, which was defined by the manufacturer as 1 unit of CSF-1. The sample was assayed by Drs E. R. Stanley and J. W.Pollard using the bone marrow colony forming assay (Stanley, 1985) which only detects biologically active CSF-1. The manufacturer's unit was found to be equivalent to 1.70 Stanley units (1 Stanley unit ≈ 0.44 fmole CSF-1 or 12 pg pure protein). The specific activity of the R&D preparation is therefore lower than a pure preparation. A stock solution of CSF-1 was prepared in Dulbecco's phosphate-buffered saline (dPBS; Sigma) containing 0.2% BSA. This solution was stored at −70°C in 50 μl aliquots each containing 500 ng CSF-1 or 1428 U. Dilutions were prepared in KSOM.

Preparation of culture media

The medium used in this work was KSOM (Table 1). The medium was prepared fresh for each replicate from individual stock solutions (0.1 M) with the following exceptions: sodium chloride and sodium lactate stock solutions were prepared at 1.0 M concentrations, and calcium chloride and EDTA was made at 0.171 M and 1.0 mM respectively, penicillin-G and streptomycin sulphate were prepared as one solution at concentrations of 10,000 units ml^{-1} and 5.0 mg ml^{-1} respectively, bovine serum albumin (BSA, fraction V, Cat. No. A9647, Lot 30H0665) was prepared at a concentration of 100 mg ml^{-1}. Small aliquots of stock solutions of all components were stored at −20°C, except for sodium lactate, sodium pyruvate and glucose, which were prepared fresh immediately before use. One day before the start of each experiment, 10 ml of each medium containing the selected amount of CSF-1 was prepared by adding all components to culture tubes (Fisherbrand 14-956-6B, Fisher Scientific, Pittsburgh) in the order listed in Table 1. Double glass-distilled water was added to bring the final volume in each tube to 10 ml. Each tube was vortexed gently and the contents were drawn into a disposable syringe (Beckton-Dickinson, Lincoln Park, NJ) and filtered through a 0.2 μm filter (Millipore, Bradford, MA) into a second tube.

Embryo collection

Mice were killed by cervical dislocation between 15.30 and 16.00 hours on day 1. Pronuclear-stage zygotes were flushed from each excised oviduct with 0.1 ml FHM (Lawitts and Biggers, 1993). The zygotes from several donors were pooled, and then washed through one drop of FHM containing 0.65 mg ml^{-1} hyaluronidase (Sigma) to remove any cumulus cells, followed by washes through two to three drops of FHM.

Embryo culture

Zygotes were cultured in 60 mm tissue culture dishes (Corning 25010, Corning, NY) in 50 μl microdrops of medium KSOM with or without the addition of CSF-1. Each culture dish contained four drops of the medium placed under 5 ml silicone oil [dimethylpolysiloxane (DMPS), 50 centistokes, Sigma]. The dishes were made up the day before the start of each replicate and they were equilibrated overnight in an atmosphere of 6% carbon dioxide, 5% oxygen and 89% nitrogen in plastic culture modules (Billups-Rothenberg, Del Mar, CA) placed inside a humidified incubator at 37°C. Five to seven zygotes were transferred from FHM into one of the drops of the medium in the equilibrated culture dishes using a mouth-controlled glass pipette with a flame-polished tip. Any remaining FHM in the transfer pipette was discarded and the pipette was refilled from another drop of culture medium. The zygotes were then transferred into the second drop and, using an identical procedure, the zygotes were passed through a third drop and finally transferred to the fourth drop. The culture dishes containing the zygotes were returned to the culture chamber at 37°C, which was gas-equilibrated and sealed. The culture modules containing the embryos were re-equilibrated with the gas mixture at 24 hour intervals throughout the culture period.

In describing the experiments, zero time is when the hCG injection was given. For simplicity, day 3 means 72 hours post hCG or 48 hours of culture, day 4 means 96 hours post hCG or 72 hours of culture, and day 5 means 120 hours post hCG or 96 hours of culture.

Some cultures were left undisturbed until either day 4 or 5. Others were left undisturbed until day 3, at which time the embryos were transferred by serial washing (see above) into fresh medium with or without CSF-1. During intervals when culture dishes were outside the culture chamber and not on the microscope stage, they were maintained at 37°C in a bench-top incubator (Baxter, Bedford, MA). On days 4 and 5 the embryo morphology, developmental progress and numbers of embryos reaching the blastocyst stage were recorded. An embryo was classified as a cleavage stage (an embryo that contained two or more cells but had not undergone compaction), a morula (a compacted embryo), or a blastocyst (an embryo possessing a visible blastocoele). At the end of the culture period (day 5) a dish of blastocysts was selected at random from each experimental group, using a table of random permutations, and stained for enumeration of the number of TB and ICM cells.

Differential fluorescent staining of TB and ICM cells

Blastocysts were stained using the method described by Ebert et al. (1985) and Papaioannou and Ebert (1988). Embryos, 3-5 at a time, were transferred from culture drops to acid Tyrode's solution, under constant observation, for 5-15 seconds until the zonae pellucidae were completely dissolved. The embryos were then removed and passed through three successive rapid washes in Ham's F-12 containing 10% calf serum (Sigma), and then to antiserum (R-105: rat anti-mouse prepared against mouse whole spleen) diluted 10% in Ham's F-12 for 30 minutes at 37°C. The blastocysts were then transferred through three successive 5 minute washes in Ham's F-12 (containing 10% calf serum) then to 10% guinea pig complement (Gibco Laboratories, Grand Island, NY), 10 μg ml^{-1} bisbenzimide and 10 μg ml^{-1} propidium iodide (Sigma) in Ham's F-12 for 30 minutes at 37°C. This

Table 1. Composition of KSOM

Component	Concentration (mM)
NaCl	95.0
KCl	2.5
KH_2PO_4	0.35
$MgSO_4$	0.20
Lactate	10.0
Pyruvate	0.20
Glucose	0.20
Glutamine	1.00
BSA	1 g l^{-1}
EDTA (tetrasodium salt)	0.01
$NaHCO_3$	25.0
$CaCl_2$ (dihydrous)	1.71
Penicillin	100 U ml^{-1}
Streptomycin-SO_4	5.0 μg ml^{-1}

procedure fluorescently stains the ICM nuclei blue and the TB nuclei pink. To count differentially stained nuclei, embryos were removed individually from complement solution to acetone-washed microscope slides, squashed under coverslips and viewed using a Zeiss epifluorescence microscope with a 365 nm band pass exitation filter and a 420 nm long pass barrier filter.

Embryo transfer

CF-1 ♀ × B6D2F1 ♂ embryos were cultured 4 days in KSOM (96 hours post hCG) with or without 540 U ml^{-1} of CSF-1. The CD-1 females were made pseudopregnant by mating them with vasectomized CD-1 males. Cultured embryos were transferred to their uteri on day 3 of pseudopregnacy; ten females each received 4-5 embryos per uterine horn, with control embryos in one horn and CSF-1 exposed embryos in the other. Fetuses were harvested from the pregnant mice on day 13 for gross examination of development and the fetuses and placentas were dissected and weighed.

Statistical analysis

Three experiments were done to determine the concentration response lines. In experiment 1, the concentrations ranged from 20 to 540 U ml^{-1}. The range of concentrations in experiments 2 and 3 was modified to 60 to 4860 U ml^{-1}.

All data were initially analyzed using graphical data exploratory analysis with the Axum program (TriMetrix, Seattle, WA). Since the percentages of blastocysts observed on days 4 and 5 are correlated, repeated measures of the response, the results are presented as bivariate plots. Fetal and placental weights have been presented as notched box plots, which indicate the 10th, 25th, 50th (median), 75th, 90th percentiles, and outliers. The notches are constructed using the formula: median±1.57×(75th%tile-25th%tile)/n. If the notches do not overlap, the medians are considered significantly different (Kadadar, 1985).

The cell count data were analysed by analyses of variance using the Number Cruncher Statistical System (Jerry L. Hintze, Kaysville, UT). The lowest order polynomial equation (linear or quadratic) needed to fit the data was computed by generalized linear interactive modelling (McCullagh and Nelder, 1989) using the GLIM System (The Numerical Algorithms Group Ltd., Downers Grove, IL). Let a quadratic equation be given by $y = b_0 + b_1x + b_2x^2$. If b_1 is significantly positive and b_2 is significantly negative, the equation has a significant maximum at $x=-b_1/2b_2$.

The categorical data were analyzed either as contingency tables by Fisher's exact method using StatXact Turbo (Cytel, Cambridge, MA) or by logistic regression analysis (Hosmer and Lemeshow, 1989) using LogExact Turbo (Cytel, Cambridge, MA).

RESULTS

Acceleration of blastocyst formation by CSF-1

The percentages of zygotes that developed into blastocysts in the presence of different concentrations of CSF-1 are shown in Table 2 and Fig. 1. Three independent experiments were done. In the first, groups of zygotes were exposed for 5 days to a range of concentrations of CSF-1 from zero to 540 U ml^{-1}. In the second and third experiments, the concentrations ranged from zero to 4860 U ml^{-1}. Observations on the incidence of blastocysts were made on day 5 in all experiments (Table 2) and on day 4 and 5 in experiment 3 (Fig. 1).

By day 5 of culture in KSOM alone, almost all zygotes developed into blastocysts. Thus, the addition of CSF-1 cannot increase the incidence of blastocyst formation at this time point. The responses to 4860 U ml^{-1} CSF-1 in the second and particularly in the third experiment, however, suggests that this high concentration inhibits the formation of blastocysts in about 25% of embryos. The probabilities that the proportions of blastocysts developing from groups of zygotes exposed to 0 and 4860 U ml^{-1} CSF-1 are equal are 0.0.086 and 0.001 in experiments 2 and 3 respectively. The percentages of blastocysts produced by day 4 in the presence of all concentrations of CSF-1, with the exception of 540 U ml^{-1} CSF-1, are approximately the same (50%). The percentage of blastocysts produced in response to 540 U ml^{-1} CSF-1 is 82.2%, which is significantly higher than the response of the control group (P=0.014). Thus, this concentration of CSF-1 accelerates the formation of blastocysts by day 4.

The effect of the concentration of CSF-1 on the numbers of TB and ICM cells in randomly selected groups of blastocysts formed by day 5 is shown in Figs 2 and 3 and in Table 3. In the first experiment, the regressions of the numbers of TB cells and ICM cells on log concentration of CSF-1 is linear. The slopes of the regression lines for the TB cells and ICM cells is 21.28 (s.e.m. 4.07, d.f. 18) and 2.13 (s.e.m. 2.34, d.f. 18) respectively. Thus the increase in numbers of TB cells as the concentration of CSF-1 increases is highly significant (P=0.00006) while the increase in ICM cells is not significantly different from zero (P=0.373) over a concentration range of 20 to 540 U ml^{-1}. The second and third experiments used wider concentrations of CSF-1. In both experiments, the linear and quadratic coefficients of the quadratic regressions of the number of TB cells on log concentration of CSF-1 are significantly positive and negative, respectively (Table 3). Thus both equations have a maximum. In experiment 2, the maximum response was obtained with an estimated concentration of 514 U ml^{-1} CSF-1 and, in experiment 3, a concentration of 815 U ml^{-1} CSF-1. As in the first experiment, there is comparatively little effect of the concentration of CSF-1 on the numbers of ICM cells.

Time of action of CSF-1

The results of a preliminary experiment suggested that CSF-1 can produce its effects when added to the culture medium on

Table 2. The number of blastocysts that develop from zygotes in KSOM in the presence of different concentrations of CSF-1 by day 5

Concentration of CSF-1 (U ml^{-1})	Experiment 1	Experiment 2	Experiment 3
0 (Control)	43/43 (100%)*	33/36 (91.7%)	33/34 (97.1%)
20	21/21 (100%)	–	–
60	21/21 (100%)	30/32 (93.8%)	35/38 (92.1%)
180	28/28 (100%)	25/25 (100%)	38/39 (97.4%)
540	29/30 (96.7%)	24/25 (96%)	42/45 (93.3%)
1620	–	26/26 (100%)	29/33 (87.9%)
4860	–	16/21 (76.2%)	18/29 (62.1%)

*No. blastocysts/total no. cultured (%)

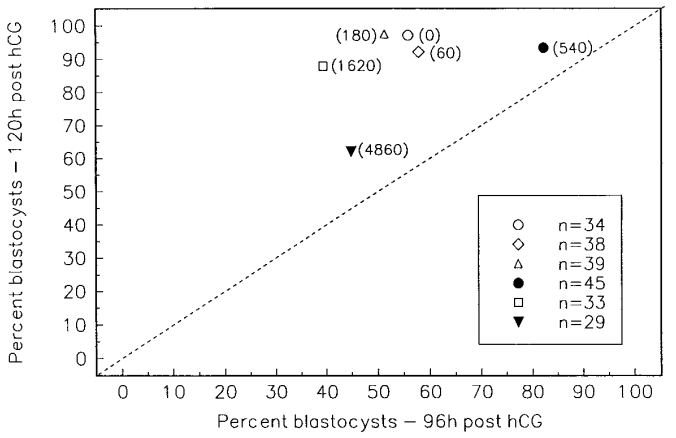

Fig. 1. A bivariate plot of the percentages of blastocysts produced by 96 hours and 120 hours post hCG of culture in different concentrations of CSF-1in experiment 3. The numbers in parentheses are the concentrations of CSF-1.

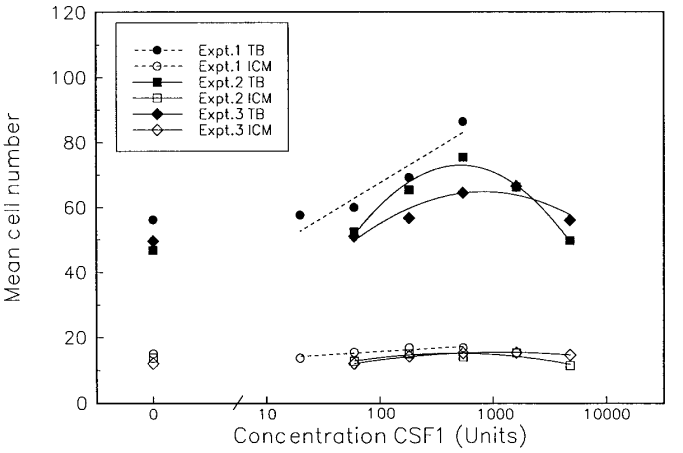

Fig. 3. The estimated quadratic regressions (solid lines) of the mean numbers of TB and ICM cells on log concentration of CSF-1 in experiments 2 and 3 compared to the linear regressions (dashed lines) found in experiment 1. The data points refer to the means of groups of blastocysts.

day 3. However, the result could also have been due to the effects of disturbing the embryos when adding the CSF-1. Six treatments were compared in an experimental design to distinguish these two variables. Two control groups of embryos were cultured undisturbed for 120 hours post hCG either without CSF-1 [denoted (0)] or in the presence of 540 U ml^{-1} CSF-1 [denoted (540)]. Two other groups of embryos were similarly exposed to CSF-1 [denoted (0,0),(540,540)], but the embryos were disturbed by renewing the medium on day 3. Two remaining groups of embryos were exposed to 540 U ml^{-1} CSF-1 either on days 1-3 [denoted 540,0)] or days 4-5 [denoted (0,540)], and were thus disturbed during the culture period. Two independent experiments were done. Observations on the incidence of blastocyst formation were made in both, but observations on the numbers of TB and ICM cells were only obtained in one.

Since the incidence of blastocyst formation in both experiments was similar the results have been pooled. A bivariate plot of the numbers of blastocysts formed by day 4 and day 5 of culture in each treatment group is shown in Fig. 4. A comparison of the incidence of blastocysts that developed in groups exposed to treatments (0) and (0,0), and also (540) and (540,540), shows that disturbing the embryos reduces the rate of blastocyst formation on both day 4 and day 5 (day 4: P=0.004; day 5: P=0.021). Nevertheless, a comparison of the incidence of blastocysts that developed in groups exposed to treatments (0) and (540), and (0,0) and (540,540), demonstrate that 540 U ml^{-1} CSF-1 signifcantly increases the rate of development of blastocysts even if the embryos are disturbed (day 4: $P<10^{-4}$; day 5: P=0.008). Exposure of the embryos to 540 U ml^{-1} CSF-1 for days 1-3 only or for days 3-5 results in all zygotes developing into blastocysts by day 5. Almost all (91.7%) of the zygotes exposed to the cytokine for days 1-3 formed blastocysts by day 4. However, significantly fewer (51.0%; $P<10^{-4}$) of the zygotes exposed to the cytokine for days 3-5 developed into blastocysts by day 4. The latter result differs from that obtained in the preliminary experiment where all of the zygotes developed into blastocysts when the 540 U ml^{-1} CSF-1 was added on day 3. Thus our results do not allow the determination of an exact period of preimplantation development when CSF-1 can produce an effect on blastocoele development, but are consistent in showing that CSF-1 can produce an effect even when present for only part of the culture period.

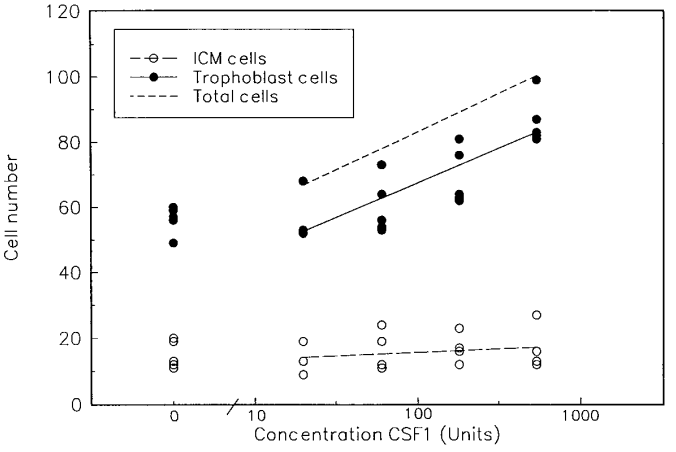

Fig. 2. The estimated regressions of the numbers of TB cells and ICM cells at day 5 on the log concentration of CSF-1 in experiment 1. The data points refer to single blastocysts. The linear regression of the total number of cells on log concentration of CSF-1 is the sum of the two estimated regression lines.

The numbers of ICM cells and TB cells on day 5 in samples of blastocysts exposed to the different regimens of CSF-1 from the second experiment is shown in Table 4. ANOVAs demonstrated highly significant differences between the numbers of TB cells that developed in response to the six treatments (P=0.0002). In contrast there were no significant differences with respect to the numbers of ICM cells (P=0.219). Disturbing the embryos on day 3 slightly depressed the number of TB cells to about the same extent in day 5 blastocysts not exposed and exposed to CSF-1 (7.25 cells; P=0.071). There were significantly more TB cells in the blastocysts produced from embryos exposed to CSF-1 compared to those not exposed to CSF-1. The increase was approximately the same in both the

Table 3. The estimated coefficients of the linear and quadratic components of the regressions shown in Fig. 3

Parameter	Experiment 2		Experiment 3	
	Linear (b_1)	Quadratic (b_2)	Linear (b_1)	Quadratic (b_2)
(A) Trophoblast				
Estimate	133.8	−24.67	68.7	−11.8
Standard error	32.9	5.95	23.9	4.39
DF	24	24	29	29
P	0.0004	0.0004	0.008	0.012
(B) Inner cell mass				
Estimate	16.45	−3.11	12.7	−2.08
Standard error	10.28	1.86	5.23	0.96
DF	24	24	29	29
P	0.122	0.108	0.022	0.039

undisturbed and disturbed groups (21.4 cells; $P<8\times10^{-6}$). The numbers of TB cells in blastocysts produced from embryos exposed to 540 U ml^{-1} CSF-1 for days 1-3 and days 3-5 were both significantly higher than in the control, disturbed embryos not exposed to CSF-1 (days 1-3: 24.4 cells, $P=2\times10^{-6}$; days 3-5: 18 cells, $P=0.00012$). In both day 1-3 and day 3-5 groups, the numbers of TB cells were approximately the same as the number in the disturbed group exposed to 540 U ml^{-1} CSF-1 throughout. Thus exposure of the embryos to CSF-1 for part of the preimplantation period increases the number of TB cells.

Fetal development following stimulation with CSF-1

8 out of 10 females became pregnant following embryo transfer. Among the pregnant females, the implantation rate for embryos cultured with and without CSF-1, 69% (n=39) and 56% (n=39)respectively, was not significantly different (P=0.254). Likewise, although the proportion of embryos that developed normally was somewhat greater among the CSF-1-treated group (33% vs 18%), the difference was not significant (P=0.131). Embryos that implanted but did not develop normally were typically represented by small moles in the uterus. Comparison of the wet weights of the fetuses and placentas of seven normal CSF-1-exposed fetuses and 13 normal control fetuses revealed no signiciant differences (Fig. 5).

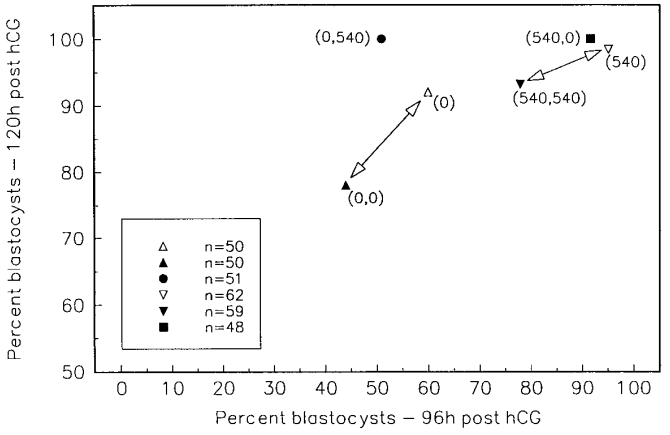

Fig. 4. A bivariate plot of the percentages of blastocysts produced by 96 hours and 120 hours post hCG under different regimens of CSF-1. The concentration of CSF-1 to which the embryos were exposed is shown as a single number or pair of numbers in parentheses beside the data point. A single number indicates that the group of embryos was exposed to this concentration from the beginning of the culture period; a pair of numbers indicates that the concentration of CSF-1 was changed from the first to the second on the day 3 of culture. Double arrows connect groups that were exposed to the same CSF-1 concentrations.

DISCUSSION

The combined results show unequivocally that exogenous CSF-1 can stimulate the development of preimplantation embryos in vitro by accelerating the formation of the blastocyst cavity and increasing overall embryonic cell number through a differential increase in the number of TB cells, with no significant effect on the number of ICM cells. The maximum response of embryos was obtained around a concentration of 540 U ml^{-1} CSF-1 when expressed in manufacturer's units, or 918 U ml^{-1} when expressed in Stanley units. This concentration of CSF-1 is very similar to the concentration that produces maximal proliferative effects in cultures of murine peritoneal exudate macrophages (Stanley and Guilbert, 1981). Futhermore the cytokine can produce the same effects even if it is present in the medium for only a part of the culture period. This in vitro stimulation of preimplantation development with CSF-1 is compatible with continued normal development in vivo.

Table 4. The mean number of TB and ICM cells on day 5 in blastocysts cultured from zygotes exposed to different CSF-1 treatments

Treatment	*n*	No. of TB cells		No. of ICM cells	
		Mean	s.e.m.*	Mean	s.e.m.*
0**	5	51.2	4.0	15.7	1.3
0,0	5	42.6	4.0	14.4	1.3
540	6	71.2	3.7	17.0	1.2
540,540	6	65.3	3.7	13.7	1.2
0,540	5	60.6	4.0	17.2	1.3
540,0	5	67.0	4.0	16.8	1.3

*Based on the pooled error mean square with 26 D.F. TB cells 80.4; ICM cells 8.3.

**The treatment is defined in terms of the concentration of CSF-1 to which the embryos were exposed.

(x) - undisturbed; (x,y) - disturbed on day 3 when the medium is replaced

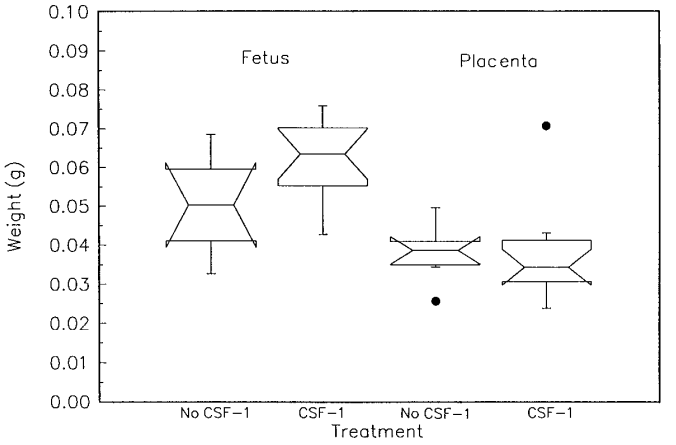

Fig. 5. Notched box plots of the fetal and placental weights of 13 day old fetuses produced by the transfer of control and CSF-1 treated embryos into pseudopregnant surrogate mothers. Number of conceptuses: No CSF-1, 7; CSF-1, 13.

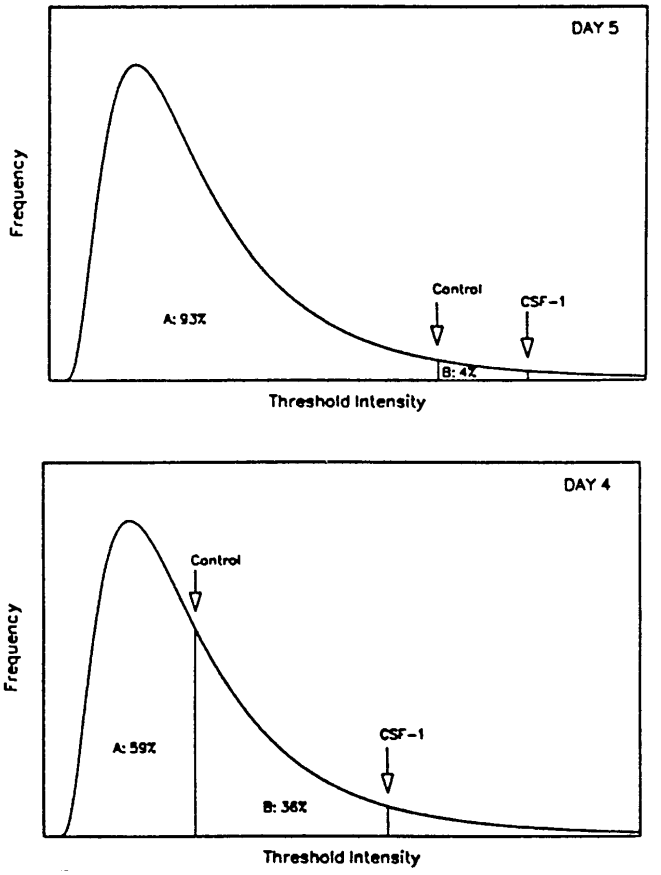

Fig. 6. A lognormal probability distribution function representing the hypothetical variation of threshold concentrations of substance X needed for blastocyst formation in the absence (control) or presence of CSF-1. Area A is the proportion of blastocysts which develop by the day 4 or day 5 in KSOM without CSF-1. Area B is the additional proportion of blastocysts which develop when CSF-1 is added to the medium.

The increase in number of TB cells over the number present in the controls is about 20 cells, or approximately 30 percent of the average number of cells in control blastocysts cultured in KSOM alone. This differential mitogenic stimulation of TB cells by CSF-1 results overall in 6-7 cell divisions taking place during the period of culture compared with 5-6 in KSOM alone. Taken together with our previous work, these results indicate that the addition of CSF-1 to KSOM brings the number of TB cells closer to the number expected in in vivo developing embryos, although the number of ICM cells is still well below that observed in embryos developing in vivo (Erbach et al., 1994). The basis for the differential effect of CSF-1 on TB cells is not known but is assumed to occur through the CSF-1 receptor. Transcripts for this receptor (the product of the c-*fms* proto-oncogene) are detected throughout preimplantation development by RTPCR (Arceci et al., 1992) and the presence of a functional receptor has been indirectly implicated by the responsiveness of embryos to CSF-1 in vitro (Pampfer et al., 1991 and the present study). Although no localization studies have been done on earlier stages, in situ hybridization for the detection of mRNA expression indicates that the receptor is expressed uniformly over the ICM and TB of 4.5 day implanting blastocysts *in vivo* (Pampfer et al., 1993). It may be that at earlier stages the receptor is present at relatively higher levels on the outside cells destined to become trophoblast than on the inner cells destined to become ICM. Alternatively, TB cells may respond differently to CSF-1 than ICM cells, or, the cytokine may simply be excluded from reaching the inner cells once tight junctions form at the time of compaction.

Exogenous CSF-1 is not required for the formation of blastocysts in vitro. In our control cultures, approximately 95 percent of zygotes developed into blastocysts. In the preliminary results of Pampfer et al. (1991), 54 percent of 2-celled embryos developed to blastocysts in medium M6 (presumably M16) without the addition of CSF-1, and early work of Whitten and Biggers (1968) showed that blastocysts would form from zygotes *in vitro* in a cytokine-free medium. Nevertheless, the addition of CSF-1 to cultures significantly increases the percentage of embryos that form blastocysts by day 4. Pampfer et al. (1991) observed the same effect on both day 4 and day 5 of culture using a less supportive medium. The combined results clearly show that CSF-1 accelerates blastocyst formation.

The diagnosis of a blastocyst relies on sufficient fluid accumulating inside the embryo to form a recognizable blastocoele. The process of fluid accumulation, which is independent of cell number (Tarkowski and Wroblewska, 1967; Smith and McLaren, 1977; Fernàndez and Izquierdo, 1980) depends on complex morphological and physiological changes (see Rodriguez-Boulan and Nelson, 1989, for a review), including the formation of a permeability seal between the outer cells of the embryo and the priming and maintenance of the sodium pump that transports water across the trophoblast (see Biggers et al., 1988, 1991, for reviews). It is possible that CSF-1 is involved in regulating the synthesis of threshold level(s) of some substance(s) critical to the development of these morphological and physiological features of the preimplantation embryo. As a working hypothesis, let us assume that blastocyst formation requires a threshold concentration of a substance X, and that CSF-1 in some way stimulates the rate at which this threshold is reached. We assume also that this

threshold varies from embryo to embryo following a lognormal distribution (Gaddum, 1945) (Fig.6). By day 4, 59 percent of the embryos acquire the threshold amounts of substance X (area A under the curve); if exogenous CSF-1 is present, a further 36 percent of embryos are stimulated to reach their critical thresholds (area B under the curve). By day 5, almost all embryos have made threshold amounts of substance X, and only a few remain that can be stimulated by CSF-1 to reach their threshold. This model could explain how CSF-1 accelerates development by stimulation of the more slowly developing embryos.

CSF-1 was discovered through its ability to stimulate division of macrophages *in vitro*. It is polyfunctional, shown by (1) the enhancement of cell survival *in vitro*, (2) the stimulation of cell proliferation, (3) the control of differentiation commitment, and (4) the enhancement of the functional activity of mature cells (Metcalf, 1988, 1991). Our results demonstrate that two of these functions occur in the mouse preimplantation embryo. CSF-1 stimulates cell proliferation and can accelerate the differentiation and function of the trophoblast, the first epithelium to develop in the mammalian embryo. Thus, our results support the suggestion of Pampfer et al. (1991, 1993) that maternal CSF-1, which is expressed at high levels in the uterus by day 3 of pregnancy (Bartocci et al., 1989), may directly mediate synchrony between the uterus and embryo around the time of implantation. As pointed out by Pampfer et al. (1993), however, the dissection of this phenomenon will be complex since CSF-1 could also exert its effects indirectly through other uterine cells such as macrophages.

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