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Effects of the Supernatants of Mixed Lymphocyte Cultures and Decidual Cell Line Cultures on Mouse Embryo Development in Vitro

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The effects of supernatants of human mixed lymphocyte cultures (MLC), with or without human decidual cell line culture extract (decidual factor; DCF), on F_1-hybrid mouse embryo development in vitro from the two-cell stage were investigated. The development of mouse embryos from the two-cell stage through the expanded blastocyst stage was facilitated significantly by the addition of supernatants of not only MLC, but also MLC supplemented with DCF (MLCDCF) to the culture medium. Moreover, the supernatant of MLCDCF accelerated the attachment of the hatched blastocyst to the culture dish substratum and the outgrowth of trophoblasts in vitro. The findings indicate that the supernatant of MLCDCF facilitates the in vitro activity of mouse embryos for implantation and that the maternal immune response, along with the decidual tissue, contributes to the implantation processes.

KEY WORDS: mixed lymphocyte reactions; decidual factor; mouse embryo development.

INTRODUCTION

Fetus implantation is a successful model of semiallograft transplantation. It has been suggested that the decidual tissue plays an important role in protecting the fetus from maternal immunological rejection in early pregnancy (1,2). Recently, a soluble factor (decidual factor; DCF) from the supernatant of human decidual cell line cultures was reported to suppress mixed lymphocyte reactions (MLR) (3). Some investigators, however, suggest that the maternal immune response is necessary or beneficial for pregnancy maintenance (4–6). In humans, couples with common human leukocyte antigen (HLA) are more prone to miscarriages than those with different HLAs (7–10). In the present study, the effects of supernatants of human mixed lymphocyte cultures (MLC) and MLC supplemented with DCF on embryo development were examined using a mouse embryo culture system in order to elucidate the role of the decidual tissue and the effect of the maternal immunological response on the implantation of an embryo.

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MATERIALS AND METHODS

Preparation of Decidual Factor (DCF)

The supernatants of a TTK-1 cell line culture derived from human decidual tissue were used as decidual factor (DCF) (3). Two types of DCF were used. The first, hereafter designated FCSDCF, was the supernatant of a TTK-1 cell line cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS). The other, designated SFMDCF, was cultured in serum-free medium composed of 5% DME/F-12, 10 m*M* Hepes, and 1.2 g/liter $NaHCO_3$ supplemented with transferrin, insulin, monoethanolamine, and sodium selenite (3). These two types of DCF were dialyzed using Whitten's medium (11) for 48 hr at 4°C and supplemented with 3 mg/ml of bovine serum albumin (BSA) (Sigma, St. Louis, MO) just before use.

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Assessment of Human Two-Way MLC in Three Different Media

RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% FCS and Whitten's medium supplemented with either 10% FCS or 3 mg/ml of BSA were used as MLC, with or without a 25% DCF solution. The lymphocytes were isolated from heparinized peripheral blood obtained from two healthy volunteers, by centrifugation using Ficoll–Paque (Pharmacia, Inc., Piscataway, NJ), and were suspended in each medium. Culturing was carried out by a two-way method using round-bottomed microtiter plates (Nunclon, Nunc, Denmark). Each well contained 5×10^4 of each of two kinds of lymphocytes (total, 1×10^5 cells/well) in a total volume of 100 μl of medium. Each sample was prepared in triplicate. Cultures were incubated for 72 to 148 hr at 37°C, in an atmosphere of 5% CO_2 in air. One-half microcurie of ^{3}H-thymidine (sp act, 6.7 Ci/mmol; NEN Research Products, Boston) was added to each well at 72, 120, and 148 hr of incubation. After culturing for another 16 hr, the cells were harvested on filter papers using a semiautomatic cell harvester (Labo Science, Tokyo) and the radioactivity was counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA) for 1 min.

Preparation of the Culture Media for the Cultivation of Mouse Embryos

(1) Whitten's medium supplemented with 3 mg/ml of BSA was used as the control medium and for both the culturing of lymphocytes and the dilution of the supernatants of the following cultures. All media were prepared using water purified through a Millipore water purification system (13).

(2) A concentration of 5×10^5 cells/ml peripheral lymphocytes, taken from one person, was cultured for 120 hr and the supernatant was collected.

(3) The supernatant of the two-way MLC was collected after culturing for 120 hr with 5×10^5 cells/ml lymphocytes (2.5×10^5 cells from each person).

(4) The two-way MLC with 5×10^5 cells/ml lymphocytes were carried out in Whitten's medium containing 25% (v/v) of serum-free medium decidual factor (SFMDCF) for 120 hr, and the supernatant was collected.

(5) The lymphocytes of 5×10^5 cells/ml, taken from one person, were cultured with concanavalin A (Con A; 30 μg/ml; Sigma, St. Louis) for 24 hr. The Con A-stimulated cells were washed and recultured for an additional 48 hr. The supernatant was then collected. All five supernatants were diluted with an equal volume of Whitten's medium and used for the mouse embryo cultures after sterilization through a 0.22-μm Millipore filter (Millipore Co., Bedford, MA).

(6) The SFMDCF solution was dialyzed for 48 hr at 4°C using two batches of Whitten's medium. It was then diluted with Whitten's medium to 12.5% (v/v) according to the concentration of SFMDCF contained in the culture supernatant mentioned in item 4. BSA, 3 mg/ml, was added to this solution and it was then sterilized.

Culturing of Mouse Two-Cell Embryos

Two-cell embryos were collected from female F_1-hybrid mice (C57Black × C3H) since the in vitro two-cell block phenomenon may have interfered with the embryo development data (11). At 4 weeks of age, superovulation was induced in F_1-hybrid mice by intraperitoneal injections of 7.5 IU pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Hormon Mfg., Tokyo) and 7.5 IU human chorionic gonadotropin (hCG; Gonatropin, Teikoku Hormon Mfg., Tokyo) 48 hr apart. Each female was caged with fertile mature ICR-strain mice immediately after the hCG injection and checked for vaginal plugs on the following morning. The day a vaginal plug was detected was considered as Day 1 of pregnancy. On Day 2 of the pregnancies, two-cell embryos were recovered from the oviducts by flushing with a small amount of medium through a 33-gauge blunt needle (12). Recovered embryos which were morphologically normal were transferred into 0.4 ml of the various media, and kept under mineral oil in a four-well chamber dish (Multidish 4, NUNCLON, Nunc, Denmark), and cultured for 120 hr. The embryos were examined under an inverted microscope and the development to expanded blastocyst and hatching blastocyst, the attachment to the culture dish substratum, and the trophoblastic outgrowth were observed. A "hatching blastocyst" is deemed to occur when more than half of the oocytoplasm is herniated from the zona pellucida. "Attachment" is defined as occurring when the trophoblastic cells around the hatched blastocyst partially adhere to the dish substratum. The condition where trophoblastic cells adhere to

the dish substratum all around the embryo is designated trophoblastic outgrowth.

Statistical Analysis

Results were statistically analyzed using a chi-square test.

RESULTS

Assessment of the Medium Used for the Mixed Lymphocyte Culture (MLC)

The addition of 25% (v/v) FCSDCF solution significantly suppressed mixed lymphocyte reactions (MLR) in RPMI 1640 supplemented with 10% FCS (Fig. 1A). The MLR in Whitten's medium supplemented with 10% FCS or 3 mg/ml of BSA were similar to those in RPMI 1640 (10% FCS). Even in Whitten's medium, SFMDCF suppressed the MLR in the same manner as FCSDCF (Figs. 1B and C). Based on these results, all subsequent lymphocyte cultures were carried out in Whitten's medium supplemented with 3 mg/ml of BSA, which was suitable for the mouse embryo cultures. Also, since SFMDCF was found to suppress the MLR as well as FCSDCF, SFDCF was used throughout the remaining experiments with the mouse two-cell embryo cultures.

Effects of the Supernatants on the Development of Mouse Two-Cell Embryos

The development of mouse two-cell embryos to either expanded blastocysts or hatching blastocysts in 50% (v/v) lymphocyte culture supernatant was no different from that in Whitten's medium alone; however, a significantly higher percentage of mouse two-cell embryos reached both the expanded and the hatching blastocyst stages in the presence of supernatants of MLC (Fig. 2a), and/or MLC with DCF, and SFMDCF solution compared with Whitten's medium alone.

Regarding the hatching process, the supernatant from the Con A-stimulated cultures also resulted in significantly higher development. Moreover, attachment to the culture dish substratum and trophoblastic outgrowth of the hatched blastocyst appeared to be significantly accelerated by the addition of the supernatant of MLC supplemented with DCF to the embryo culture medium (Fig. 2b) compared with the development of embryos cultured in Whitten's medium alone ($P < 0.001$ and $P < 0.05$), the supernatant of MLC alone ($P < 0.01$ and $P < 0.01$), and SFMDCF solution ($P < 0.001$ and $P < 0.05$). The phenomenon of trophoblastic outgrowth of mouse embryos was induced only in the presence of the supernatant of MLC supplemented with DCF (Table I).

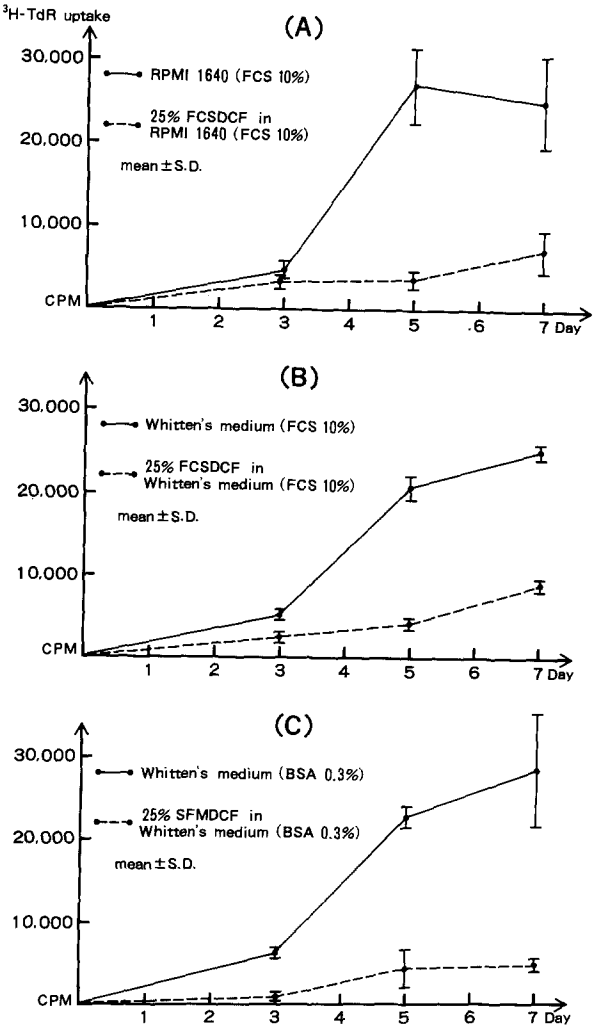

Fig. 1. The reactivities of mixed lymphocytes with and without decidual factor, indicated by ^{3}H-thymidine uptake, in the three different media. In every medium, mixed lymphocyte cultures were reactive; however, two kinds of decidual factors effectively suppressed the reactions as shown in A, B, and C.

DISCUSSION

Previously, the maternal immunological reaction was thought to be detrimental to early embryo de-

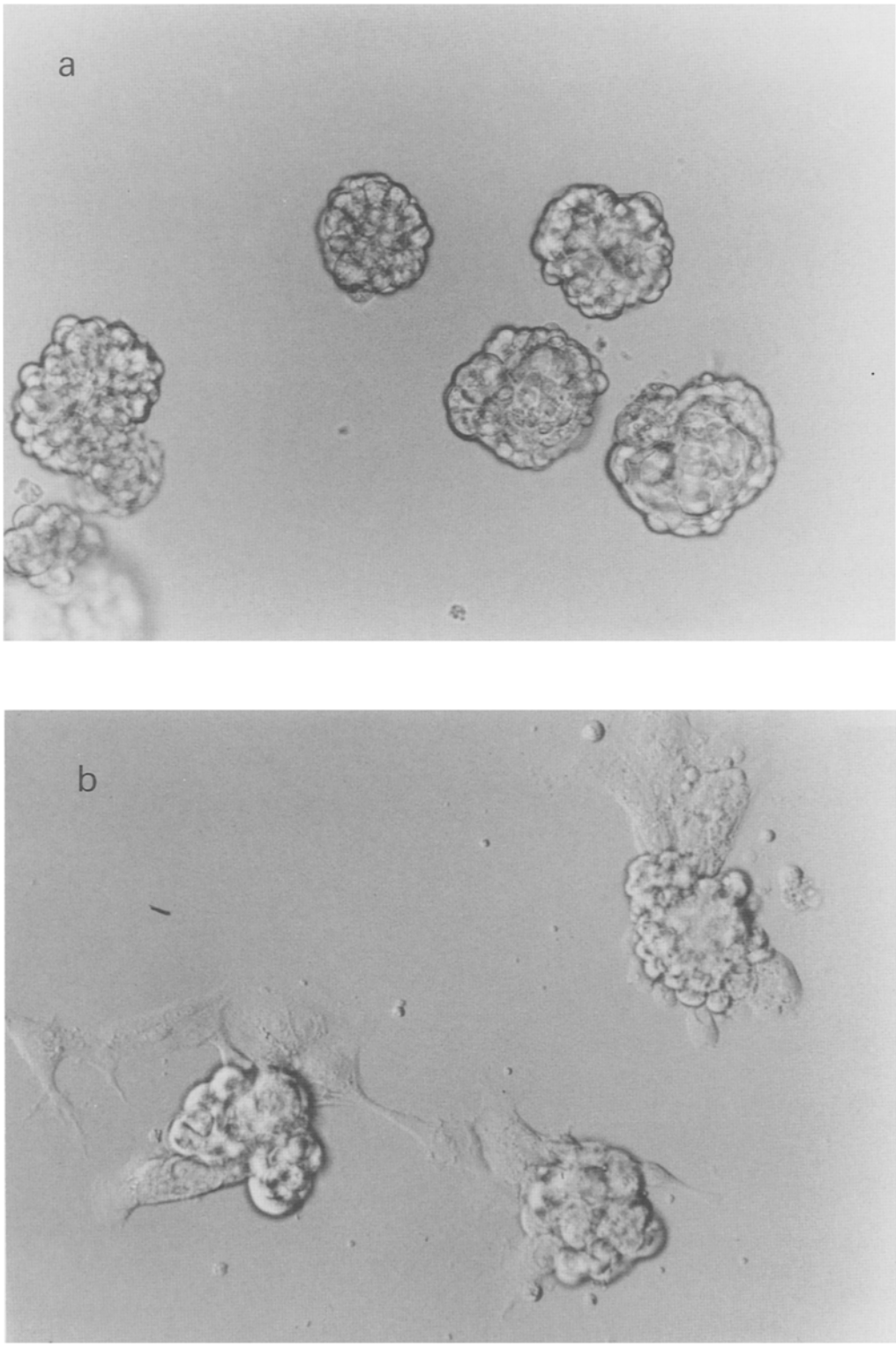

Fig. 2. Microscopic observation of hatched mouse blastocysts developed from the two-cell stage, cultured in vitro for 120 hr. (a) The blastocysts cultured in MLC medium without DCF. These blastocysts did not attach to the dish substratum. (b) The three attached hatched blastocysts in MLC medium supplemented with DCF.

Table I. Two-Cell Mouse Embryos Cultured in Whitten's Medium and Five Test Media Containing Four Types of Supernatants and One Solution*

Culture medium	No. of embryos cultured	No. of embryos that developed at each stage (%)			
		Expanded blastocyst	Hatching blastocyst	Attachment	Trophoblastic outgrowth
Whitten's medium	126	107 (84.9)	69 (54.8)	0 (0)	0 (0)
Lymphocyte culture supernatant in Whitten's medium (50%, v/v)	31	25 (80.6)	18 (58.1)[b]	0 (0)	0 (0)
Con A-stimulated lymphocyte culture supernatant in Whitten's medium (50%, v/v)	89	82 (92.1)	77 (86.5)[b]	0 (0)	0 (0)
SFMDCF solution in Whitten's medium (12.5%, v/v)	69	69 (100)[a]	58 (84.1)[b]	0 (0)	0 (0)
MLC supernatants in Whitten's medium (50%, v/v)	52	51 (98.1)[a]	50 (96.2)[b]	1 (1.9)[c]	0 (0)
MLC with SFMDCF (25%, v/v) supernatant in Whitten's medium (50%, v/v)	58	57 (98.3)[a]	53 (91.4)[b]	21 (36.2)[b,d,f]	5 (8.6)[b,e,g]

* Superscripts a, b, and c represent significant differences between Whitten's medium and the other five groups. (a, $P < 0.05$; b, $P < 0.01$; c, not significant). Superscripts d and e represent significant differences between MLC with DCF and MLC (d, $P < 0.001$; e, $P < 0.05$). Superscripts f and g represent significant differences between MLC with DCF and DCF solution (f, $P < 0.001$; g, $P < 0.05$).

velopment based on the fundamental concepts of conventional immunology regarding transplantation (14,15). Recently, however, it has been suggested that the incompatibility of mutual HLAs in a couple produces a successful pregnancy because of the induction of blocking antibodies and/or suppressor cells by the active immune response of maternal lymphocytes to fetal alloantigens (16). Furthermore, decidual tissue has been found to have an immunosuppressive effect by secreting some factors (3,17). There are, however, only a few reports concerning the effects of lymphokines produced by maternal lymphocytes on embryonal development and implantation. Hill *et al.* described the inhibitory effects of supernatants of mouse and human lymphocyte cultures, stimulated with PHA, Con A, LPS, or mixed lymphocyte culture (MLC) on the development of CD-1 mouse embryos from the two-cell stage to the late blastocyst stage. It was reported that these inhibitions were caused by monokines and lymphokines (18). These results lead to the hypothesis that some spontaneous abortions may be caused by the factors released from lymphocytes during maternal immunological rejection. Although different strains of mice were used in the present study and the study described above, this difference does not account for the fact that the present study yielded opposing results. F_1-hybrid mice were used in this study instead of CD-1 or other inbred strains because the embryos from the F_1 hybrid have a very low rate of incidence of in vitro two-cell block phenomenon. Only the supernatants of MLC facilitated F_1-hybrid mouse embryo development from the two-cell stage to the hatching blastocyst stage. Moreover, the supernatant of MLC with DCF induced further development from the expanded blastocyst stage to the attachment or trophoblastic outgrowth stage in vitro. Based on these data, it is suggested that lymphokines secreted in the maternal immune response to the embryo in the decidual tissue environment may play an important role in implantation. Therefore, it is suggested that lymphokines released from maternal lymphocytes and factors secreted from decidual tissue synergically enhance embryonal development and implantation. Our results agree with another report which found that the addition of T cell-derived lymphokines stimulated the proliferation of murine placental cells in the fetus in vitro (19). Embryos of different strains, however, may respond differently to the supernatant of MLC as reported by Hill *et al.* The results of the present study suggest that the immunological reaction promoted embryo development and that some factors from the decidual tissue plays a role in implantation. In our previous studies, decidual factor (DCF) suppressed the proliferation phase and possibly the differentiation phase during MLC, suggesting that cytotoxic T cells against the embryos were inhibited by DCF (3). The present study supports the hypothesis that the maternal immune reaction promotes embryo development and that successful implantation occurs as a result of a local immune reaction. The decidual cell line is cultured extensively to obtain a large

amount of DCF and to identify the material by purification of the DCF.

In human in vitro fertilization and embryo transfer (IVF-ET) therapy, the success rates of implantation and the viability of the in vitro fertilized embryos still remain very low. The results of this study imply that a means which would elevate the maternal immune reaction to fetal antigens (HLA or trophoblastic antigens) may be helpful not only in preventing immunological habitual abortions but also in increasing the implantation rate in the human IVF-ET program.

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