In vitro Studies on PGC or PGC-Like Cells in Cultured Yolk Sac Cells and Embryonic Stem Cells of the Mouse

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Summary. The present study aims: 1) to determine those conditions which promote the proliferation of primordial germ cells (PGCs) of the migratory phase in the yolk sac; and 2) to examine the effects of yolk sac cells as a feeder layer—under the conditions mentioned above—upon the embryonic stem (ES) cells (R1) with high potential for entering the germ line in vivo in chimeras. In murine yolk sac cells obtained on Day 10.5-11.5 of pregnancy and cultured in a modified Dulbecco's modified Eagle's medium (DMEM-plus/20: the postfix represents the concentration of FBS added in percentage), many cells exhibited strong immunoreactivities to the monoclonal antibodies 4C9 and 2C9 which are known to react with PGC specifically. Both the 4C9- and the 2C9-positive cells were sensitive to the treatment with busulfan added in vitro, supporting the supposition that they were PGCs. The respective numbers of the 4C9- and the 2C9-positive cells increased approximately 4 and 12 times when they were cultured in DMEM-plus/20 fortified with SCF, LIF, bFGF and TNF-α (DMEM-N/20). When the R1 cells were cultured in the yolk sac-conditioned DMEM-N/20 medium on the laminin substratum, the entire colonies were faintly stained with 4C9 but not with 2C9. At times solitary ES cells migrated out from the colonies, and reacted strongly with 4C9. When yolk sac cells and R1 cells were cultured on the two sides of a collagen-coated membrane, the yolk sac cells being feeder cells, some R1 cell colonies were intensely stained as a whole with either the 4C9 or the 2C9 antibody, suggesting that these colonies might be composed of cells clonally derived from stem cells which either had been destined to become the germ line cells or had already acquired cellular characteristics close to PGCs. It was tentatively concluded that the R1 cell population contained, as judged from the immunoreactivities, germ-cell-like cells, and that the yolk sac cells and/or their secretory products might facilitate the proliferation of, or the conversion of R1 cells to, the germ-cell-like cells.

Embryonic stem (ES) cell lines of mice (EVANS and KAUFMAN, 1981; MARTIN, 1981), clonal cell lines derived from pre-implantation mouse embryos, retain the toti- or pluri-potency even after 250 passages (SUDA et al., 1987). When ES cells are conjoined with blastomeres of early embryos, they are capable of being integrated into the host embryonic cell population and differentiate into all cell lineages including the germ line (e.g., BRADLEY et al., 1984; GOSSLER et al., 1989), giving rise to fertile chimeric mice. The pluri-potent ES cells have provided valuable tools in the experimental embryology of mice by allowing the genetic manipulation of cells in vitro, e.g., gene targeting or transgene integration, and examination of the effects of genetic modifications upon phenotypes in vivo in the offspring of the germ-line chimeras (ROBERTSON, 1987; JOYNER, 1993).

The usefulness of the pluri-potent ES cells, however, is not limited to basic biological research but extends into new perspectives of applied fields of life sciences. For example, attempts are being made in medicine to produce transplantable human tissues in vitro from ES cells (O’SHEA, 1999; THOMSON and ODORICO, 2000). In agricultural science, on the other hand, much hope is placed in ES cells as a versatile tool to produce genetically-engineered farm animals; the process will be greatly facilitated if germ cells can be generated in vitro from genetically modified ES cells.

Since ES cells contribute to all somatic cell lineages and to the germ line in vivo in chimeras, it should be theoretically possible to induce them to differentiate in vitro into any particular type of cell in the body, including germ cells. So far, ES cells have been shown to be capable of differentiating in

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vitro into skeletal muscle cells (ROHWEDEL et al., 1995), cardiac muscle cells (UNO, 1982), neurons (BAIN et al., 1995; FRAICHARD et al., 1995), hematopoietic cells (SCHMITT et al., 1991; WILES and KELLER, 1991; MIWA et al., 1991) and keratinocytes (BAGUTTI et al., 1996). The question still remains, however, whether ES cells could give rise to cells of the germ line in vitro. As one approach to this problem, we examined the effect of yolk sac cells as a feeder layer upon ES cells under conditions where the proliferation of primordial germ cells (PGCs) is facilitated; yolk sac cells constitute the natural cellular environment of PGCs of the migratory phase.

Using alkaline phosphatase as a marker in mouse embryos, PGCs are first identifiable as a small cluster of cells at the posterior end of the primitive streak on about 7 day post coitum (dpc) (GINSBERG et al., 1990). They then move into an area of the endoderm which forms the allantois and the yolk sac (e.g., ROUGHI, 1990); at 10 dpc they migrate into the dorsal mesentery, and finally into the developing genital ridges. By 11.5 dpc most of the PGCs have completed entry into the genital ridges, which have already begun to differentiate into the gonads (CLARK and EDDY, 1975; DE FELICI et al., 1992). During this period, PGCs proliferate, increasing in number from approximately 150 on 8.5 dpc to about 10,000 on 12.5 dpc (TAM and SNOW, 1981).

Using an in vitro culture system, GODIN et al. (1990) demonstrated that soluble factors released by developing gonads may stimulate the proliferation of PGCs. Later, several purified growth factors were shown to affect PGC numbers in vitro. One of these factors, identified when using cultured murine PGCs, was the stem cell factor (SCF) (MATSUI et al., 1991; DOLCI et al., 1991; GODIN et al., 1991), which is a product of the murine Steel (Sl) locus and serves as a ligand for the c-kit receptor protein encoded by the W locus (CHABOT et al., 1988; GEISSLER et al., 1988; WITTE, 1990). The leukemia inhibiting factor (LIF), a cytokine which was originally identified as a factor responsible for the regulation of differentiation in hematopoietic cells (GOUGH and WILLIAMS, 1989), has been found to play essential roles in the maintenance of toti- or pluri-potency in ES cells (WILLIAMS et al., 1988; SMITH et al., 1988) as well as in their survival, and under certain conditions, the proliferation of mouse PGCs in culture (MATSUI et al., 1991; DE FELICI and DOLCI, 1991; PESCE et al., 1993). It has also been shown that the addition of basic fibroblast growth factor (bFGF) to the PGC culture media causes the transformation of PGCs to ES cell-like cells, i.e., embryonic germ cells (EG cells) (MATSUI et al., 1992; RESNICK et al., 1992; MUMMERY et al., 1993). Transforming growth factor-β (TGF-β), on the other hand, negatively regulates the proliferation of PGCs (GODIN and WYLIE, 1991). KAWASE et al. (1994) further reported that tumor necrosis factor-α (TNF-α) stimulates the proliferation of PGCs in culture without transforming them into EG cells; the effect is specific to the PGCs before and during their migration to the presumptive gonads.

More recently, interleukin-4 (IL-4), a pleiotropic cytokine, was added to the list of substances which are capable of stimulating an increase in the number of mouse PGCs cultured in vitro, although IL-4 appears to act as a survival factor rather than a mitogen for PGCs (COOKE et al., 1996).

In the present series of experiments, we first attempted to establish those culture conditions which allow the proliferation of PGCs of migratory phase in the yolk sac, and second to examine the possible effects of yolk sac cells as a feeder layer in vitro upon the ES cells with the experimentally-confirmed high potential to enter into the germ line in vivo in chimeras.

MATERIALS AND METHODS

Animals

Mice of ICR strain were used throughout the experiments. They were purchased from Sankyo Lab (Tokyo) and were kept under regulated temperature (25°C) and illumination cycles (14 h light and 10 h darkness) with access to food and water ad libitum until sacrifice. The day when copulatory plugs were found was designated as Day 0.5 of pregnancy.

Culture media

The basic synthetic medium used throughout the present study for the culturing of either ES cells or yolk sac cells was Dulbecco’s modified Eagle’s medium (DMEM) (high glucose type; GIBCO BRL, Rockville, MD) added with 0.1 mM non-essential amino acids (0.1 mM; GIBCO BRL), sodium pyruvate (1 mM; Wako, Osaka), 1-glutamine (2 mM; Wako), β-mercapto-ethanol (0.1 mM; Sigma, St Louis, MO), penicillin (100 units/ml; Meiji-seika, Tokyo), streptomycin (50 μg/ml; Meiji-seika), and nucleosides (adenosine, 30 nM; guanosine, 30 nM; cytidine, 30 nM; uridine, 30 nM and thymidine, 10 nM; Sigma). This medium will be referred to below as DMEM-plus. When DMEM-plus was supplemented with fetal bovine serum (FBS; Lot No. 5D2073, JRH Biosciences, KS), the percentage of the serum added was denoted by the postfix, e.g., DMEM-plus/10 for
DMEM-plus medium fortified with 10% FBS and DMEM-plus/20, if 20% FBS was added.

For the induction of PGC proliferation, the following cytokines and growth factors were added to DMEM-plus/20: SCF (32.5 ng/ml, mouse recombinant; Sigma), LIF (1000 units/ml, mouse recombinant; Gibco BRL), bFGF (20 ng/ml, human recombinant; Sigma), and TNF-α (4 ng/ml, human recombinant; R & D systems, MN). This medium will be referred to as DMEM-NT/20. Although the membrane-associated form of SCF has been known to be more effective in supporting the survival of PGCs than the soluble form (Dolci et al., 1991; Matsui et al., 1991), we used the readily available soluble form of SCF. The concentrations of the cytokines and growth factors were initially chosen on the basis of data published in the literature (see Introduction) and subjected to minor modifications according to the results of pilot experiments.

The yolk sac-conditioned medium was prepared by culturing the yolk sac cells in DMEM-plus/10 for about 4 days (1 day after confluence was reached). The medium was collected by decantation and filtered through an Acrodisc filter unit (Gelman Sciences, MI).

Collection of yolk sac cells:
To collect yolk sac cells, pregnant female mice were killed by cervical dislocation on Day 10.5, 11 and 11.5. The uteri were slit open and fetuses were carefully removed with the yolk sac and placenta. The excised specimens were placed in Ca²⁺-, Mg²⁺-free phosphate-buffered saline (PBS(-)), and yolk sacs were removed from the fetuses under a dissecting microscope. Approximately 15 yolk sacs were pooled in 10 ml of the 0.05% trypsin-EDTA solution, and incubated at 37°C in a water bath with gentle shaking for 20-30 min. The cells were dissociated by pipetting, and the resultant suspension was passed through a nylon mesh unit (Falcon #2350, Becton Dickinson) to remove tissue debris, diluted with 15 ml of DMEM-plus/10, and centrifuged at 600 x g for 5 min. The pellet was resuspended in DMEM-plus/10 (12 ml for cells equivalent to 15 yolk sacs of Day 10.5; 18 ml for Day 11.0; 24 ml for Day 11.5).

Culture of yolk sac cells:
Circular cover slips (15 mm in diameter; Matsunami, Tokyo) coated with type I collagen were placed in each well of a 6-well plate (#430343; Corning Costar). The coating procedures were as follows. About 800 µl of 0.3% type I collagen solution (#1PC-13, Koken, Tokyo) was pipetted into each well of the 6-well plates containing the coverslip and left at room temperature overnight. The collagen solutions were decanted and the wells were dried at room temperature for approximately 5 h in a clean bench under UV illumination till complete dryness was attained. A 2 ml aliquot of the yolk sac cell suspension was pipetted into a single well of the 6-well culture plates at an approximate density of 4.5 x 10⁶ cells/well. Incubation was carried out at 37°C for approximately 3 days under a humidified atmosphere of 5% CO₂ in air, until the cells reached confluence.

Busulfan treatment of yolk sac cell monolayer culture:
Yolk sac cells prepared from Day 10.5-11.5 embryos of ICR mice were seeded onto collagen-coated cover slips in the wells of the 6-well plates (about 4 x 10⁵ cells/well) and cultured in DMEM-plus/20 for 3 days under standard conditions. When the culture became confluent, the medium was changed to fresh DMEM-plus/20, augmented with the stock solution of busulfan dissolved in DMSO (2 µl/well), and cultured for another 24 h. The final concentrations of busulfan ranged from 0.05-500 µg/ml.

ES cells:
A murine ES cell line, R1 (Nagy et al., 1993), originally derived from 129/Sv embryos, was kindly provided by Dr. Andras Nagy (Samuel Lunenfield Research Institute, Mount Sinai Hospital, Toronto, Canada). The high potentials of R1 cells to contribute to the germ line in chimeras have been experimentally confirmed (Nagy et al., 1993). The ES cells were cultured in collagen-coated plastic dishes (60 mm in diameter; Falcon #3002, Becton Dickinson) at 37°C under an atmospheric condition of 5% CO₂ in air, on a feeder layer of mitomycin-C-inactivated (10 µg/ml at 37°C for 2-2.5 h) embryonic fibroblasts (EMFI) prepared from Day 13.5-15.5 embryos of ICR mice according to the procedures described by Robertson (1987). DMEM-plus/20 was supplemented with murine LIF (1000 units/ml) (recombinant; Gibco BRL) and used for the culture. For passage, the cells were briefly washed with PBS(-), placed in a trypsin-EDTA solution (0.05% trypsin dissolved in Tris-buffered saline (pH 7.5) containing 0.02% EDTA), and incubated for about 3 min under an atmosphere of 5% CO₂ in air. The detached cells were harvested, suspended in 5 ml of DMEM-plus/20 medium, and centrifuged at 200 x g for 5 min. The pellet was resuspended in the LIF-supplemented DMEM-plus/20. The cells were then seeded onto a feeder layer of mitomycin-treated EMFI in the 60 mm Falcon dishes and incubated at 37°C under a humidified atmosphere of 5% CO₂ in the air.
**Culture of ES cells on yolk sac cells**

R1 cells cultured on the EMFI feeder cells were trypsinized with the 0.05% trypsin-EDTA solution and centrifuged at about 500 × g for 5 min. The pellet was resuspended in DMEM-plus/20 and seeded onto a non-coated 60 mm Falcon dish without a collagen coat. They were cultured at 37°C for 1 h under an atmosphere of 5% CO₂ in air. The non-adherent cells, which mostly consisted of the ES cells, were collected by aspirating the medium and placed in a 50 ml centrifuge tube. The yolk sac feeder cell layer was rinsed once with fresh medium. The suspension of non-adherent cells was centrifuged and the pellet was resuspended in DMEM-NT/20. The non-adherent cells were added to the yolk sac feeder layer.

![Image of yolk sac feeder cell culture](image.png)

**Fig. 1.** Schematic illustration of the 'double-sided' culture method of ES cells and yolk sac cells on the collagen-coated membrane filter. **A.** 150 μl of a yolk sac cell suspension in DMEM-plus/10 was placed onto the collagen-coated membrane filter. **B.** After incubation at 37°C under an atmosphere of 5% CO₂ in air till the cells reached confluence, the collagen-coated membrane filter units were turned upside down, and 150 μl of ES cell suspension in the DMEM-NT/20 was placed onto the collagen-coated membrane filter.

**Culture of ES cells on the laminin substrate**

The collagen-coated cover slips were double-coated with laminin (Biomedical Technologies, MA) as follows. Frozen laminin solution (2.2 mg/ml in 0.05 M Tris, 0.15 M NaCl, pH 7.4) was thawed at 4°C and diluted with a buffer (pH 7.5; 0.05 M Tris-HCl, 0.2 M NaCl) to give a final concentration of 38 μg/ml. One milliliter of the laminin solution was pipetted (4 μg/cm²) into each well of the 6-well plates and left at room temperature for 1 h. The supernatant was removed by aspiration and the plates were dried at room temperature.

After the removal of adherent cells as described above, the non-adherent ES cells were seeded onto the laminin-coated cover slips placed in one well of 6-well plates at an approximate density of 4.5 × 10⁴ per well and cultured for 4 days in a mixture of the yolk-sac conditioned medium, fresh DMEM-plus, and FBS (1:1:0.5); growth factors (SCF, LIF, bFGF and TNF-α) were added to the mixture immediately before initiating the culture (the final concentrations of the growth factors were the same as those in the DMEM-NT/20 medium).

**Culture of ES cells on Matrigel basement membrane matrix**

Circular cover slips (15 mm in diameter; Matsunami, Tokyo) were placed in each well of the 6-well plates as done for the collagen coating, and 88 μl of Matrigel solution (Becton Dickinson) were added onto each cover slip and incubated at 37°C for 1 h.

After the removal of adherent cells from the trypsinized R1 cultures on the EMFI feeder layer, as described above, the ES cells were seeded onto the thin Matrigel layer at a density of 0.9–1.0 × 10⁴ per well and cultured for 2 days in the 1:1:0.5 mixture of the yolk-sac conditioned medium, fresh DMEM-plus, and FBS. The cytokines and growth factors (SCF, LIF, bFGF TNF-α) were added to the mixture immediately before initiating the incubation (the final concentrations were the same as those in DMEM-NT/20). The cells were cultured for 2 days under standard culture conditions.

**'Double-sided culture' on collagen-coated membrane filter**

A collagen-coated filter chamber (CCM-24, Okken Cellgen, Tokyo) was put upside-down (Fig. 1) into each well of a 24-well plate (430262, Corning Coster). Since the membrane of the chamber was coated by the manufacturer only on the top side, we coated the bottom side of the membrane by treating it with collagen solution as described above. About 150 μl of
yolk sac cell suspension (about $5 \times 10^4$ cells/ml) in DMEM-plus/10, prepared from 10.5–11.5 day embryos of ICR mice, was placed onto the newly-coated side of the membrane filter, i.e., the bottom side of the membrane which was now facing upward. After yolk sac cells reached confluence, the collagen-coated membrane filters were turned upside down so that the right side was up and 150 $\mu$l of the DMEM-NT/20 was pipetted into the chamber. Subsequently, 150 $\mu$l of R1 cell suspension ($5 \times 10^4$ cells/ml) was added and cultured for 2 days (Fig. 1).

**Immunofluorescence microscopy of PGCs**

For the identification of PGCs, the monoclonal antibodies 4C9 (rat IgM) (a kind gift from Mitsui-Seiyaku, Tokyo) and 2C9 (mouse IgG plus IgM $\alpha$-light chain; a kind gift from Prof. Takao NISHIDA, Department of Anatomy and Physiology, Collage of Agriculture and Veterinary Medicine, Niho University, Fujisawa-shi) were used. The 4C9 antibody, which recognizes a Le$^a$ cell surface antigen, is a reliable marker of mouse PGCs during the migration period and after their arrival at the fetal gonads (YOSHINAGA et al., 1991). The 2C9 antibody was established by fusing myeloma cells (X63-Ag8-653) with the spleen cells immunized with sexually indifferent gonads derived from 6-day old chick embryos (MAEDA et al., 1994). The 2C9 antibody has been shown to detect specifically PGCs from the migratory stage to the stage of sexual differentiation in chick embryos (MAEDA et al., 1994). Our preliminary data show that the antibody specifically react also to the PGCs of the mouse (unpublished data); the reactivity of 2C9 to PGCs was shown by the sensitivity of the 2C9-positive cells to busulfan as will be described in the Results. For immunocytochemical studies, the cells were grown on glass cover slips (culture grade, Matsunami, Tokyo) placed in one well of the 6-well plates. After the decantation of the medium and two washings with PBS(–), the cells were fixed with 4% paraformaldehyde dissolved in PBS(–) for 10–15 min. The fixed cells were permeabilized with 0.1% Triton X-100 (Nacional Tesque, Kyoto) in PBS(–) for 10–15 min and afterwards washed with
Fig. 3. Fluorescence photomicrographs showing cells with 4C9 and 2C9 immunoreactivities in the yolk sac cells cultured in the DMEM-NT/20 medium. A and C. Phase contrast photomicrographs. B and D. Fluorescence photomicrographs of the same fields as those shown in A and C, respectively. The number of both the 4C9-positive (B) and the 2C9-positive cells (D) increase strikingly compared with the yolk sac cells cultured in the DMEM-plus/20 medium. Scale bar: 50 μm

4 changes of fresh PBS(-). For blocking of the non-specific reaction, the specimens were soaked overnight in 1% bovine serum albumin (BSA) solution in PBS(-). The blocking solution was removed by aspiration and the primary antibody solution (1:150 dilution for 4C9 and 1:750 dilution for 2C9) was added. Incubation was done for 2 h at room temperature or overnight at 4°C. At the end of incubation, the primary antibody solution was removed, and the specimens were washed with PBS(-) 5 times. As the secondary antibodies, fluorescein-5-isothiocyanate (FITC)-conjugated anti-rat IgM μ chain goat IgG fraction (cat. no. 55749, lot no. 39035, Organon Teknika, Cappel Research Products, NC) and tetramethylrhodamine-isothiocyanate (TRITC)-conjugated antimouse IgG3 goat IgG (cat. no. 1100-03, lot no. H664-V364C, Southern Biotechnology Associates, AL) were employed. Both of the secondary antibodies were used at a 1:100 dilution. After incubation for 1-2 h at room temperature, the cells were washed several times with PBS(-). The cover slips with the cells were removed from the wells and mounted on a slide glass with glycerol. Observations were made using an Olympus model IMT-2 microscope equipped with a fluorescence microscopic assembly.

RESULTS

PGC like cells in monolayer culture of yolk sac cells

In the yolk sac cells cultured in DMEM-plus/20, both the 4C9- and the 2C9-immunoreactive cells could be clearly detected after immunocytochemical staining with the respective antibody (Fig. 2). Both the 4C9- and the 2C9-positive immunoreactive cells exhibited characteristic morphological appearances distinctly different from the majority of yolk sac cells when they were examined under a phase contrast microscope: they were round in shape and often appeared darker than the yolk sac cells (Fig. 2A, B). When the yolk sac cells were cultured in DMEM-NT/20, i.e., DMEM-plus/20 supplemented with the
Fig. 4. Mean densities of the 4C9- and the 2C9-positive cells in the yolk sac cells cultured using two different types of culture media. A. Yolk cells cultured in DMEM-plus/20 medium. B. Yolk sac cells cultured in DMEM-NT/20 medium. Vertical bars indicate the standard deviation.

cytokines and growth factors, striking increases in the numbers of both the 4C9-positive and the 2C9-positive cells took place (Fig. 3). The densities of the 4C9-positive and the 2C9-positive cells were quantitatively assessed under the fluorescent microscope when the yolk sac cell cultures reached confluence on Day 4 of culture. In the yolk sac cell cultures using DMEM-plus/20, the mean density of 4C9-positive cells was 130 ± 17 (mean ± standard deviation) cells/cm² (n = 5) and that of 2C9-positive cells, 58 ± 22 (mean ± s. d.) cells/cm² (n = 4); 1 cm² of the yolk sac cell culture at confluence contained approximately 7.5 × 10⁵ cells on average. The density of 4C9-positive cells was invariably greater than that of 2C9-positive cells; the difference was statistically significant at p < 0.05 (Fig. 4a).

On the other hand, in the yolk sac cell cultures in DMEM-NT/20, the mean density of 4C9-positive cells was 444 ± 21 (mean ± s. d.) cells/cm² (n = 4) and that of 2C9-positive cells, 715 ± 47 (mean ± s. d.) cells/cm² (n = 4). In contrast to the results of a previous series of experiments where the cells were cultured in DMEM-plus (Fig. 4a), the density of 2C9-positive cells was always higher than that of 4C9-positive cells (p < 0.01) (Fig. 4b).

Effects of busulfan upon the number of 4C9- or the 2C9-positive cells

The results of experiments described in the previous section indicated that the 4C9- and the 2C9-immunoreactive cells present in the yolk sac cell culture were probably PGCs of the migratory phase. In order to confirm our supposition, we examined the effects of busulfan in vitro upon the 4C9-positive as
well as the 2C9 positive cells in the yolk sac cells cultured in DMEM-plus/20. The concentration range of busulfan examined was between 0.1 to 300 μg/ml (Fig. 5).

The effect of busulfan upon the number of 4C9-positive cells became noticeable at a concentration of 1 μg/ml; as the concentration was increased, the density of 4C9-positive cells decreased sharply (Fig. 5). The density of 2C9-positive cells also decreased in a fashion similar to that of 4C9-positive cells after the treatment with busulfan (Fig. 5). The yolk sac cells, on the other hand, were not affected by the treatment with busulfan within the concentration ranges examined, as judged by the morphological appearance and the capacity of the cells to remain adhered to the substratum.

### The 4C9- and the 2C9-positive cells in the ES cell population

The previous section has presented evidence indicating that the 4C9- and the 2C9-immunoreactive cells in the yolk sac cell cultures represent PGCs of the migratory phase, and that DMEM-NT/20 is capable of stimulating the proliferation of these cells. The next attempt was to examine: 1) whether or not the ES cell population, i.e., R1 cells, contain cells with immunoreactivities similar to PGCs, and 2) if such cells are present, how they are influenced by the yolk sac cells or the yolk sac-conditioned medium under the conditions which stimulate the proliferation of PGCs.

When R1 cells were cultured in DMEM-plus/20
either on the laminin substratum without EMFI feeder cells, few cells were strongly 4C9- or 2C9-positive. Although the ES cell colonies were faintly stained with 4C9, no colonies reacted with 2C9.

We then cultured R1 cells on a laminin substratum, without the EMFI feeder layer, in the yolk sac-conditioned DMEM-NT/20 medium. The ES cells formed colonies of a rounded shape, and the entire colonies were faintly stained with the 4C9 antibody, whereas they hardly reacted to the 2C9 antibody; similar to the ES cells cultured in DMEM-plus/20. However, cells migrating out from the colonies were often seen to react strongly with the 4C9 antibody (Fig. 6); the density of these strongly 4C9-positive cells was $13 \pm 4$ cells/cm$^2$ ($n=3$). Although there were a few solitary cells reacting strongly with the 2C9 antibody, the number of positive cells was extremely small ($1 \pm 0.2$ cells/cm$^2$, $n=3$).

In the R1 cell population cultured in the yolk sac-conditioned DMEM-NT/20 on Matrigel, the 4C9-positive and the 2C9-positive cells were seen scattered in the culture (Fig. 7). Under a phase contrast microscope, the immunoreactive cells appeared round in shape and somewhat dark in contrast. The densities of 4C9- and 2C9-positive cells were estimated by fluorescence microscopy; both 4C9-positive and 2C9-positive cells were detected at an approximate density of 30-40 cells/cm$^2$.

Influence of yolk sac cells upon ES cells across the collagen-coated membrane filter

In this series of experiments, yolk sac cells and R1 cells were cultured on the two sides of a collagen-coated membrane. After 2 days of culture, R1 cells were fixed and immunocytochemically stained with the 2C9 or the 4C9 antibody. In some colonies, the entire colony was intensely stained with either the 4C9 or the 2C9 antibody, suggesting that these colonies are composed of cells clonally derived from single stem cells highly immunoreactive to the respective antibody. Several such colonies were observed in a well (Fig. 8). The numbers of colonies stained with the 2C9 antibody were very small compared to those of the 4C9 positive colonies.

When R1 cells were cultured on EMFI feeder cells in DMEM-NT/20, no colonies appeared strongly positive to either 4C9 or 2C9 such as those mentioned above, although most of the colonies were faintly stained with the 4C9 but not with the 2C9 antibody.

DISCUSSION

The aims of the present series of experiments were: 1) to determine those conditions which promote the proliferation of PGCs of the migratory phase in the yolk sac, and 2) to examine the effects of yolk sac
cells as a feeder layer upon the ES cells with high potentials for entering the germ line in vivo in chimeras, under the conditions mentioned above.

We demonstrated that, in the culture of yolk sac cells obtained on Day 10.5-11.5 of pregnancy, 4C9- and 2C9-immunoreactive cells are present and able to undergo strong cell proliferation in the DMEM-NT/20 medium. The respective numbers of either 4C9- or 2C9-immunoreactive cells increased approximately 4 or 12 times when they were cultured in the DMEM-NT/20 instead of the DMEM-plus/20.

The monoclonal antibody 4C9 was raised against a Le^a [Galβ1→4 (Fucα1→3) GlcNAc] antigen which is expressed specifically in blastomeres of the cleavage stage embryos in the inner-cell-mass (ICM) cells of blastocysts and in PGCs (Yoshinaga et al., 1991); it has been used as a reliable marker for PGCs of the migratory phase. The other monoclonal antibody, 2C9, was produced by immunizing BALB/c mice with sexually indifferent gonads of chick embryos (Maeda et al., 1994). This antibody has been shown to specifically react with PGCs of chickens (Maeda et al., 1994), and as mentioned in the Materials and Methods, also reacts to PGCs of the mouse in vivo (unpublished data). Thus, the 4C9- and the 2C9-immunoreactive cells in the yolk sac cell cultures are highly likely to be PGCs of the migratory phase.

Our tentative identification of the 4C9-positive and the 2C9-positive cells as PGCs in the yolk sac was supported by the observation that the addition of busulfan to the medium selectively eliminated the 4C9- as well as the 2C9-immunoreactive cells. When busulfan, an anti-leukemic alkylating agent, is administered to pregnant rats during 13.5 - 20.5 and 13.5 - 16.5 dpc, it destroys the germ cells in the gonads, both the testis and the ovary, of the offspring (HEMS-
worth and Jackson, 1963a, b; Kasuga and Takahashi, 1986), owing to the selective suppression of the proliferative activities of PGCs (Hemsworth and Jackson, 1962, 1963a, b; Kasuga and Takahashi, 1986). Busulfan treatment is now being used as a reliable method to deplete germ cells of the hosts for xenogeneic spermatogonial transplantation in mice and rats (Brinster and Zimmermann, 1994; Ogawa et al., 1999).

As described in the Results, the density of 4C9-positive cells was always higher than that of 2C9-positive cells in the yolk sac cells cultured in the DMEM-plus/20 medium. Curiously, when DMEM-NT/20 was used instead of DMEM-plus/20, the density of 2C9-positive cells became invariably higher than that of 4C9-positive cells. While the 4C9 antibody is specific to murine PGCs of the migratory phase (Yoshinaga et al., 1991), the 2C9 antibody was raised against the PGCs of the genital ridge (Maeda et al., 1994). Therefore, 2C9 might preferentially stain PGCs slightly advanced in the developmental stage. If this is indeed the case, then the DMEM-NT/20 medium not only stimulated the proliferation of PGCs but also facilitated the differentiation and/or the maturation of PGCs in the yolk sac.

In the R1 cell populations cultured on the laminin substratum without any EMFI feeder layer, the colonies were faintly positive to the 4C9 antibody but not to the 2C9 antibody. Interestingly, the solitary R1 cells occasionally found around the colonies, probably representing cells which had migrated out from the colonies, were strongly 4C9 positive. It was tentatively concluded that these 4C9-positive R1 cells might be those cells which were due to enter predominantly into the germ line or had already acquired cellular characteristics close to the PGCs of the migratory phase.

Interesting observations were made when R1 cells were cultured on the collagen-coated membrane on the reverse side of which the yolk sac cell monolayer was formed. While the majority of the R1 cell colonies were stained only faintly with the 4C9 antibody, some colonies exhibited intense reaction to the antibody. Similarly, small number of colonies strongly reacted to the 2C9 antibody.

The results may be interpreted to suggest that the 4C9 or 2C9 positive colonies are clonally derived from single R1 cells which either have been destined to become the germ line cells or have already acquired cellular characteristics close to PGCs, such as those observed as solitary 4C9 or 2C9 positive cells in R1 cells cultured on the laminin or Matrigel substratum. It would be intriguing to examine the developmental potentials of individual R1 cells in the 4C9-positive or 2C9-positive colonies to give rise to the germ line cells either in vivo or in vitro, although at present it unfortunately does not appear to be technically feasible to isolate the 4C9-positive colonies selectively. If indeed the 4C9-positive colonies could be isolated, and they were found to be solely composed of cells of the germ line cell compartment in the ES cell population, the fact would be of great value from the viewpoints of both basic and applied life sciences.

In summary, it was tentatively concluded that the R1 cell population might contain the germ-cell-like cells as judged from the immunoreactivities to 4C9 and 2C9 antibodies, and that the yolk sac cells and/or their secretory products might facilitate the proliferation of, or the conversion of R1 cells to, the germ-cell-like cells. Further experimental analysis is certainly needed to understand the mechanisms underlying these curious phenomena.

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