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Adhesiveness of mouse primordial germ cells to follicular and Sertoli cell monolayers

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SUMMARY

The adhesiveness of female and male mouse primordial germ cells (PGCs) to somatic cell monolayers of various origin has been studied in the definite conditions of an *in vitro* system. PGCs were isolated from the gonads of embryos of various *post coital* ages according to the method of De Felici & McLaren (1982), and seeded on the cell monolayers. PGCs from 12·5 to 15·5 days *post coitum* (dpc) embryos specifically adhered to Sertoli and follicular cells obtained from adult gonads. The percentage of female PGCs which adhered to follicular cell monolayers was significantly higher than that of male PGCs. No significant adhesion was seen between PGCs and somatic cell monolayers obtained from various embryonic and adult tissues.

The results obtained indicate that the simple *in vitro* assay described in the present paper might help to characterize the cellular interactions between somatic and germ cells during gonadal development.

INTRODUCTION

One of the major goals of developmental biology is to determine the molecular basis of intercellular recognition and to describe how these interactions contribute to the formation of the tissues. In the early stages of development of the mammalian embryo, primordial germ cells (PGCs) migrate from the yolk sac wall into the genital ridges, the structures from which testes and ovaries will form. Although interactions between developing germ cells and somatic cells might be of great relevance to basic events that occur in PGCs during this period such as migration, proliferation and initiation of meiosis, little is known about the cellular and molecular aspects of this phenomenon.

The studies reported in the present paper are focused on the earliest events of heterologous adhesion between PGCs and monolayers of somatic cells from different sources. The results suggest that a specific adhesiveness exists between PGCs and follicular or Sertoli cells obtained from prepubertal gonads, and give some general information which might be of help for further studies aimed to identify the molecules involved in the complex cell–cell interactions that occur during the development of the gonads.

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Key words: primordial germ cells, adhesiveness, cell interaction, Sertoli cells, mouse, follicular monolayers.

MATERIALS AND METHODS

Media and culture conditions

A Hepes-buffered, serum-free medium (MH), containing 1 mg ml^{-1} bovine serum albumin (De Felici & Siracusa, 1982) was used for the isolation of all cell types. The cells were cultured in Hepes-buffered medium 199 (M199) with Earle's salts (Flow Laboratories), supplemented with freshly prepared L-glutamine (1 mM), sodium pyruvate (0·5 mM), sodium bicarbonate (15 mM). Heat-inactivated foetal calf serum (FCS, Gibco) was routinely added to M199 at concentrations of 5–10 %. The cultures were carried out, unless otherwise indicated, in the wells of Terasaki microtest plates (Falcon) containing about 20 µl of medium per well, maintained at 32 °C (Sertoli cells) or 37 °C (all other cells) in a water-saturated atmosphere of 95 % air, 5 % CO_2.

Isolation of PGCs and somatic cells from embryos

Embryos were obtained from CD-1 mice (Charles River, Italy) on the 12th–16th day of pregnancy (11·5–15·5 d*pc*). The urogenital regions (gonad and mesonephros) were collected in MH. From 12·5 d*pc* onwards the gonads were sexed by their characteristic appearance and carefully separated from mesonephros by fine needles. PGCs were directly released into M199 by pricking the gonads with a needle and collected by a mouth-operated micropipette. In order to facilitate the release of PGCs, pricking was usually preceded by incubation of the urogenital regions for 10–15 min in 0·01 % EDTA. This treatment increases the yield of PGCs released from gonads without apparently affecting their behaviour and viability (De Felici & McLaren, 1982, 1983). In control experiments no difference in adhesiveness to somatic cell monolayers was found between PGCs isolated with or without the EDTA step (results not shown). After isolation, PGCs were cultured for 1 h in a Petri dish (35 × 10) in 1·5 ml M199 under standard conditions. Control experiments had shown that at the end of the culture period the viability of PGCs was always greater than 90 %.

In order to obtain gonadal somatic cells monolayers, small fragments of the remains of the gonads were cultured in the standard conditions indicated above. In some culture wells the somatic cells, after about 4 days, formed a confluent layer with very few germ cells (identifiable by alkaline phosphatase, see below) present at the top. Only the wells which developed a confluent monolayer were used for the adhesion test.

Monolayers of mesodermic cells were prepared by cutting mesonephroi into small pieces, which were cultured under the same conditions as above. During 3–4 days the mesonephric cells migrated on the plastic substratum to form a confluent monolayer.

Preparation of blood cells, bone-marrow cells and hepatocytes

Blood cells. Blood was obtained by cardiac puncture from ether-anaesthetized mice. Separation of the blood cell types (mononuclear cells, polymorphonuclear cells, erythrocytes) was carried out in a preformed Percoll gradient according to the procedure described by Pertoft, Hirtenstein & Kagedal, (1979). After isolation the three cell fractions were washed to remove Percoll, counted and resuspended in MH to a concentration of about $5–6 \times 10^4$ cells ml^{-1}.

Bone marrow cells. A suspension of bone marrow cells was obtained by flushing with MH the diaphyses of femurs of adult mice (1ml/femur) and dispersing the marrow plug by three passages through a 22-gauge needle. After counting, the cells were centrifuged (100*g* × 5 min) and resuspended in MH to a final concentration of about $5–6 \times 10^4$ cells ml^{-1}.

Hepatocytes. The liver of an adult mouse was cut in small fragments that were aspirated many times through a 1 ml syringe, first without needle, then with a 22-gauge needle. Tissue fragments and cell clumps were allowed to settle, and the supernatant cell suspension (which contained hepatocytes, Kupffer cells and blood cells) was collected, centrifuged and resuspended to a final concentration of $1–2 \times 10^6$ cells ml^{-1}. Hepatocytes were then isolated from this suspension using a self-generated Percoll gradient (Pertoft *et al.* 1977).

Follicular and Sertoli cell monolayers

Ovaries from 10- to 12-day-old mice were collected in MH and pricked with needles to release follicles. The follicles were transferred to 2 ml of the same medium containing 1 mg ml^{-1} collagenase (Type I, Sigma). Following incubation for 15–20 min at 37 °C the follicles were set free of the oocyte by pipetting with a mouth-operated micropipette, washed three times in M199 and cultured in Terasaki wells (20–25 follicles per well). During culture the follicular cells attached to the bottom of the well and within 2 days a confluent layer was formed.

Seminiferous tubules were isolated by incubating decapsulated testes from 12- to 14-day-old mice with 1 mg ml^{-1} collagenase in MH for 20–30 min at 37 °C with gentle shaking. The tubule fragments released by this treatment were washed three times in M199, pipetted up and down several times, and cultured in Terasaki wells. 'Sertoli cells only' monolayers were obtained by removing, after 2 days of culture, the germ cells present on top of the layer of flattened Sertoli cells, by a 3 min incubation at room temperature with 20 mM Tris-HCl buffer, pH 7·4, (Stefanini *et al.*, 1980). The cells were cultured for a further 24 h before using.

Fibroblast and kidney cell monolayers

Fibroblasts were obtained from fragments of the skin of 15·5–16·5 d*pc* foetuses or of the dermis of 12- to 14-day-old mice cultured in a 35 × 10 Petri dish. After 2–3 days of culture fibroblasts spread out and were harvested by trypsinization (0·2 % trypsin, 15 min, 37 °C). They were then allowed to grow at confluency in Terasaki wells during a further 2 days of culture.

Kidney cell monolayers were obtained from freshly excised kidneys of 12- to 14-day-old mice. The cortex was chopped into small fragments, which were treated with 1 mg ml^{-1} collagenase for 40 min with gentle shaking at 37 °C. The tubule fragments released were washed three times in M199 and cultured in the standard culture conditions. The kidney cells migrated on the plastic substratum to form a cell layer which, after 2–3 days, largely covered the bottom of the wells.

All monolayers were inspected microscopically for confluency before adhesion experiments.

Adhesion assay

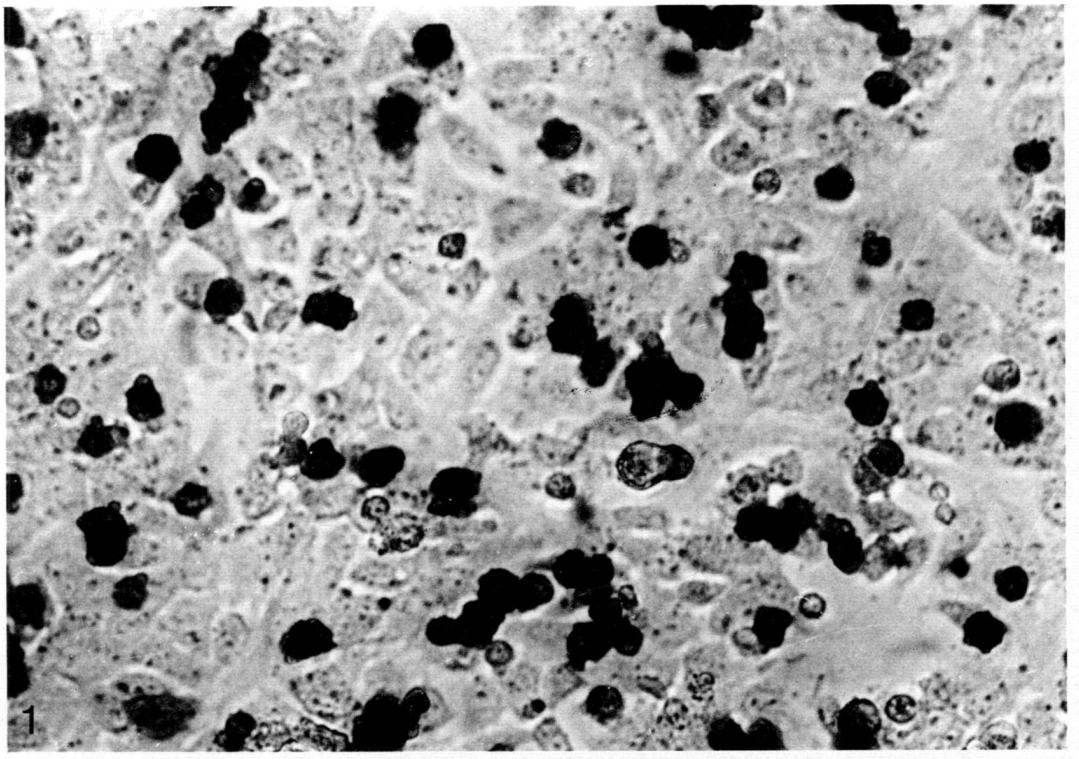
The adhesion assay is in part based on the one devised by Walther, Ohman & Roseman, (1973). The assay measures the adhesion of single cells or small aggregates (6–8 cells) to a cell monolayer. The adhesion assay was initiated by removing the medium from the well and immediately replacing it with the cell suspension to be tested.

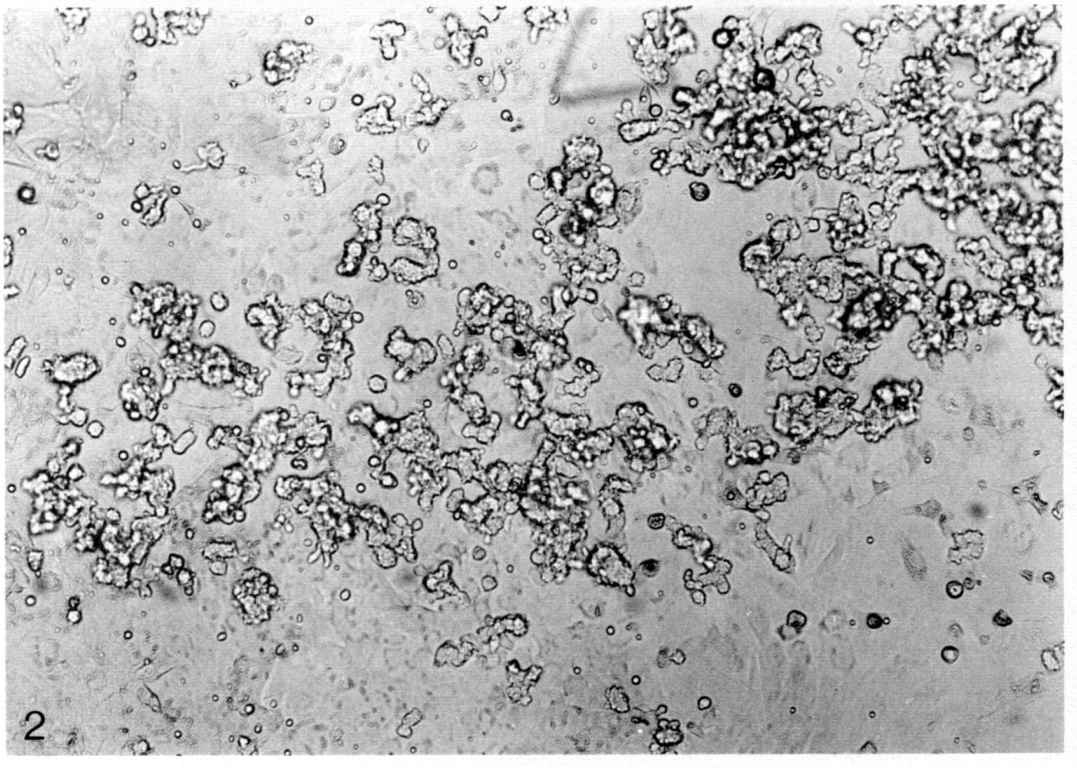
Primordial germ cells. Groups of carefully counted PGCs (usually 200–300) in suspension were picked up with a micropipette in a small volume (about 5–10 µl), rinsed in MH and added to individual Terasaki wells. After incubation (60 min, 37 °C, unless otherwise specified), the unattached cells were removed by washing three times the well with MH at room temperature. The wash medium was vigorously ejected against the side walls of the well, and immediately removed by gentle aspiration with a micropipette.

Alkaline phosphatase staining was used to identify PGCs adhering to the cell layer. To this purpose the monolayers were fixed with 9:1 alcohol–formalin for 2–3 min, washed with H_2O and stained with 0·5 mg ml^{-1} Fast blue RR in 0·25 % Naphtol, pH (8·6) (Sigma) for 30 min at room temperature. The number of stained cells (PGCs) was determined under an inverted microscope in at least two wells for each experiment (Fig. 1).

Control experiments showed that no alkaline phosphatase positive cells were present in the cell monolayers used for PGC seeding.

Blood cells, marrow cells and hepatocytes. Essentially the same method described above was used. In this case, 10 µl of the cell suspension (500–600 cells) were overlaid on the monolayer of follicular cells. After 1–2 h of culture followed by the standard washing procedure, the number of adhering cells was determined by counting the cells still attached to the monolayer. Microscopic examination showed that cells from the suspension that had attached to the monolayer were round, lying on the top of the monolayer, and readily distinguishable from the typical epithelioid, flat follicular cells.





Figs 1 & 2

RESULTS

PGC adhesion to layers of embryonic somatic cells

During a 1 h incubation, less than 10 % male and female PGCs from 13·5 d*pc* embryos attached to monolayers of somatic cells obtained from various embryonic organs (gonads, mesonephros, skin) (Table 1). By using phase-contrast microscopy, it was observed that unattached germ cells showed a greater tendency to adhere to each other than to the monolayer, and generally formed large aggregates of up to 50–100 cells. (Fig. 2).

PGC adhesion to layers of somatic cells from prepubertal animals

PGCs from 13·5 d*pc* embryos did not adhere (less than 10 %) to layers of dermal fibroblasts or kidney cells (Table 1), and their behaviour was similar to that shown on embryonal cells.

Both female and male PGCs, obtained from 12·5 to 15·5 d*pc* embryos, attached to Sertoli cells during a 1 h incubation. The adhesion rate was generally higher for female than for male PGCs (70–90 % *vs* 60–70 %, respectively, Table 1). PGCs from earlier, not sexable gonads (11·5 d*pc* embryos), had lower adhesion values (about 30–50 %, data not shown). These values were significantly different (*t* test,

Table 1. *The adhesion of female and male primordial germ cells (obtained from 13·5 d*pc *embryos) to cell monolayers of various origin during incubation for 1 h at 37 °C*

Monolayers	Adhesion	
	♀	♂
Somatic cells from 13·5 d*pc* male or female gonads	–	–
Mesodermal cells from mesonephros	–	–
Embryonal fibroblasts	–	–
Dermal fibroblasts	–	–
Kidney cells	–	–
Follicular cells	+++	from ± to ++ (see text)
Sertoli cells	+++	++

– = adhesion less than 10 %; ± = adhesion 20–30 %; ++ = 60–70 %; +++ = more than 70 %. Two or four experiments (2–4 wells per experiment) for each type of somatic cell monolayer were performed.

Fig. 1. Female PGCs from 13·5 d*pc* foetal gonads seeded on a 'Sertoli cells only' monolayer and stained for alkaline phosphatase after 1 h incubation at 37 °C. (× 450).

Fig. 2. Female PGCs from 13·5 d*pc* foetal gonads seeded on a mesodermal monolayer. PGCs do not adhere to the monolayer and form large aggregates in suspension. (× 240).

P never greater than 0·05) from the pooled (male + female) adhesion values of PGCs from each later stage (12·5–15·5 d*pc* embryos).

The attachment of PGCs to follicular cell monolayers appeared to be sex specific. Female PGCs from 12·5 d*pc* or older embryos were more adhesive than male PGCs (Table 1 and Fig. 3). Although the adhesion values of male PGCs varied between 20 % and 70 % between different experiments and as a function of the age of the embryos, a statistically significant difference with female PGCs was found in each experiment (*t* test, *P* never greater than 0·05).

PGCs from 11·5-day-old foetal gonads attached to follicular cell monolayers with

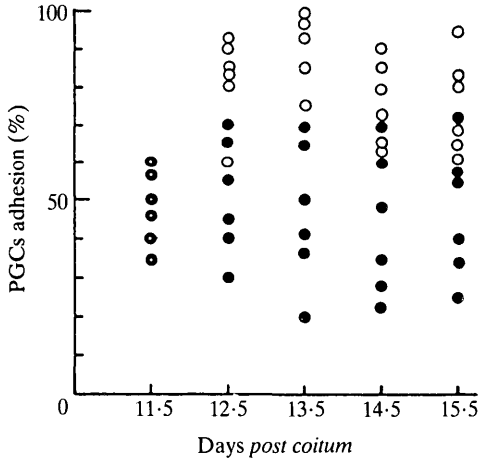


Fig. 3. Adhesion of PGCs from foetuses of various *postcoital* ages to monolayers of follicular cells after 1 h at 37 °C. Each symbol represents a determination of PGC adhesion in a single well (three different experiments). ○ female PGCs; ● male PGCs: ◐ embryos not sexable (see text).

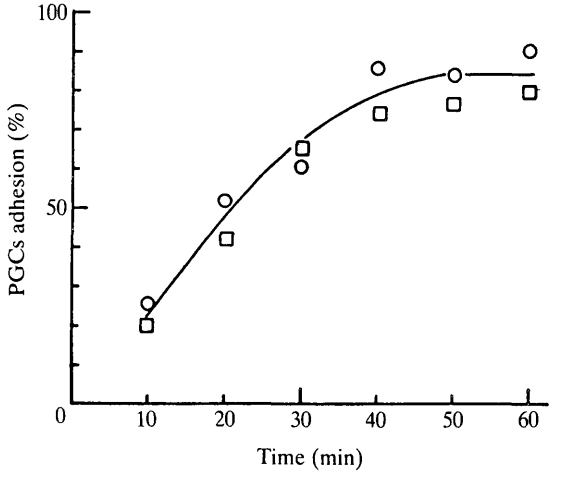


Fig. 4. Kinetics of female PGCs (from 13·5 d*pc* foetal gonads) adhesion to follicular cell monolayers at 37 °C. Each symbol is the average of the results obtained in three wells (two different experiments).

Table 2. *The adhesion of cell populations of various origin to follicular cell monolayers during 1–2 h incubation at 37 °C*

Cell population	Adhesion
Erythrocytes	–
Mononuclear cells	–
Polymorphonuclear cells	–
Bone-marrow cells	±
Hepatocytes	±

– = adhesion less than 10 %; ± = adhesion 20–30 %. Two or three experiments were performed for each type of somatic cell (2–3 well per experiment).

a lower efficiency (30–60 %) than PGCs from older embryos (Fig. 3). The difference was, however, not significant.

The kinetics of female PGC adhesion to follicular cell monolayers during the first 60 min after seeding are shown in Fig. 4. After a progressive increase during the first 40 min, the adhesion level remains essentially constant.

Adhesion of blood cells, bone-marrow cells and hepatocytes to follicular cell monolayers

The adhesion of five different cell types to follicular cell monolayers is shown in Table 2. Blood cells (mononuclear cells, polymorphonuclear cells, erythrocytes) attached in a very low percentage (less than 10 %) to the cell layer. Bone-marrow cells and hepatocytes showed a higher tendency to adhere (20–30 %).

Since different methods were used to identify adhering PGCs and somatic cells, a direct comparison between their percent adhesion values cannot be made. However, because the attached somatic cells were easily distinguishable from the flat follicular cells of the monolayer (see Methods), we are confident that the measured differences reflect real differences in adhesion properties.

Calcium and temperature-dependence of PGC adhesion to follicular cell monolayers

In the experiments in which calcium dependence was examined, follicular cell layers were washed twice with calcium-free MH immediately before addition of female PGCs, similarly rinsed in Ca^{2+}-free MH. Practically no adhesion was seen after a 1 h incubation in Ca^{2+}-free medium. During this time follicular cells changed their shape from flattened epithelioid to rounded cells. This change was, however, reversible and after 1–2 h in complete MH follicular cells recovered their normal appearance and were able to support with success the adhesion of PGC. PGCs did not show obvious morphological changes for at least 1 h in Ca^{2+}-free MH, and regained their ability to adhere to a follicular layer when transferred to complete MH.

The temperature dependence of the adhesion process was clearly demonstrated by the finding that no PGCs attached to follicular cells during a 1 h incubation at low temperature (4 °C).

Effect of trypsin, neuroaminidase and UDPgalactose on PGC adhesion to follicular cell monolayers

The treatment of female PGCs with 0·2 % trypsin (10 min, 37 °C) immediately before seeding, markedly reduced their adhesivity, to less than 30 %. We have not been able until now to study the reversibility of this effect because of difficulties in manipulating PGCs after trypsin treatment (the cells become very sticky), and because optimal culture conditions for PGCs are presently not available (De Felici & McLaren, 1983).

Neuroaminidase treatment, used to remove sialic acid from the cell surface ($2–5 \times 10^{-4}$ i.u. neuroaminidase from *Vibrio cholerae* per 10^3 PGCs, 1 h, 37 °C), did not alter PGC adhesiveness.

In order to verify a possible involvement of cell surface galactosyltransferases in PGC adhesion to follicular cell monolayers, the adhesion assay was run in the presence of the sugar nucleotide UDPgalactose (UDPGal), at 1 mM concentration. No significant reduction of adhesion was observed.

DISCUSSION

Several methods have been developed to quantitate adhesion between homologous and heterologous cells. By using *in vitro* tests similar to the adhesion assay used in the present study, heterotypic recognition and adhesion specificities have been demonstrated in many cell systems, such as between neuronal and glial cells (Sensebrenner & Mandel, 1974; Foucaud, Reeb, Sensebrenner & Gombos, 1982; Grumet, Rutishauser & Edelman, 1983), neuronal cells and myoblasts (Grumet *et al.* 1982), granulocytes and endothelial cells (Boogaerts, Yamada, Jacob & Moldow, 1982). Specific *in vitro* intercellular adhesion between rat spermatocytes and Sertoli cells has also been recently demonstrated (Ziparo, Geremia, Russo & Stefanini, 1980; Grootegoed *et al.* 1982). These studies have indicated that in systems in which heterotypic cell–cell interactions are of fundamental importance, mechanisms are developed by the cells that, as for homotypic aggregation (for reviews, see Marchase, Vosbeck & Roth, 1976; Frazier & Glaser, 1979), allow reciprocal recognition and specific adhesion.

In the present paper the adhesion of mouse primordial germ cells to various somatic cell monolayers has been analysed by a method that allows the quantitative and reproducible measurement of such interaction. Two main observations emerge from our data: first, PGCs from 12·5 to 15·5 d*pc* embryos specifically adhere to somatic cells of mature gonads (but not to other embryonal and adult cell types); second, the adhesion appears to be sex specific for male PGCs, which preferentially adhere to Sertoli cells, whereas female PGCs adhere equally well to follicular and

Sertoli cells (Table 1). Differences in cell surface carbohydrates (De Felici, 1984) and in glycoconjugates synthesis (De Felici, Boitani & Cossu, 1985) between female and male PGCs might in part explain these different adhesion properties.

The low rate of adhesion of PGCs from 11·5 d*pc* embryos compared to PGCs from later stages might be in relation to their migratory activity and/or their high mitotic rate (De Felici & McLaren, 1983; McLaren, 1981). The lack of adhesion of PGCs from all embryonic stages examined to somatic cells obtained from 13·5 d*pc* foetal gonads, suggests that PGCs acquire the molecular specificity to interact with gonad tissue long before somatic cells. A clear interpretation of this result is, however, difficult because the identity of the somatic cells from foetal gonad that form a monolayer in culture is uncertain. In addition, a strict comparison between the adhesion properties of somatic cells from adult and embryonic gonads cannot be done, because longer culture times are required to obtain confluent monolayer from the latter cells. On the other hand, the follicular cells' ability to bind PGCs does not appear to be completely specific, since about 20–30 % bone marrow cells and hepatocytes adhere to follicular cell monolayers (Table 2). This is not surprising, since cells of different origin may have different tendencies for hetero- or homotypic attachment (Walther *et al.* 1973; Prop, 1975).

The analysis of some aspects of PGC adhesion to follicular cell monolayers has revealed that, as it occurs in various other systems (for references, see Warren, Edelman & Cunningham, 1983), the adhesion between PGCs and follicular cells requires calcium ions. In addition, this cell–cell interaction appears to be mediated by specific surface molecules, since it is greatly reduced by germ cell trypsinization, and to require metabolic activity, since it does not occur at low temperature. The lack of significant effects on PGC adhesion by neuroaminidase treatment suggests that surface sialic acid residues are not involved in this phenomenon.

The possiblity that surface galactosyltransferases might mediate cellular adhesion by binding their specific substrates on adjacent cell surfaces (Shur, 1983) was examined by running the adhesion assay in the presence of UDPGal, a sugar nucleotide which forces to completion the transferase reaction, thereby dissociating the enzyme from its galactosylated substrate. The addition of UDPGal was without effect on the adhesion of PGCs to follicular cells, suggesting that surface galactosyltransferases are similarly not involved in the phenomenon.

The mechanism of cell adhesion *in vitro* is not fully understood. In short-term assays evidence has been obtained for a multistep model for cell adhesion (Weiss, 1970; Marchase *et al.* 1976; Frazier & Glaser, 1979; Foucaud *et al.* 1982). It has been proposed that initially cells recognize each other, and then other factors (such as complementary ligands, physicochemical interactions or chemiotactic effects of components released by cells) lead to cellular adhesion. In certain *in vitro* systems the high specificity of homotypic or heterotypic short-term adhesion has been correlated with the presence of distinct cell-surface glycoproteins (such as N-CAM, neuronal cell adhesion molecule or L-CAM, liver cell adhesion molecule, Edelman,

1983). In a recent study D'Agostino, Monaco, Stefanini & Geremia, (1984) have given evidence that two glycoproteins of apparent relative molecular mass 78 000 and 51 000 might be responsible for the specific adhesion of rat spermatocytes to Sertoli cells.

Owing to our ignorance of the chemical nature and detailed molecular structure of germ cell surface it would be presently unwise to do more than discuss in general terms the mechanisms of PGC adhesion. Mouse PGCs fail to adhere to various artificial or natural substrates, including plastic, glass, agar, gelatin, collagen (De Felici & McLaren, 1983), human fibronectin (M. De Felici, unpublished observation), and have the tendency to form large homotypic aggregates in suspension when cultured *in vitro* (De Felici & McLaren, 1983). Similarly, PGCs isolated from *Xenopus* embryos do not adhere to artificial substrates, but firmly attach to living cells substrates (Heasman, Mohun & Wylie, 1977).

Xenopus PGCs adhere to all cell monolayers studied (embryonic cells, adult kidney and mesentery), and the main factor responsible for adhesion appears to be the fibronectin secreted by the cell monolayers (Heasman *et al.*, 1981). It is to be noted that in the *Xenopus* studies PGCs were isolated at migratory stages and the adhesion occurred only after 2–3 days of culture. Our results with PGCs from 11·5 d*pc* foetal gonads suggest that also in the mouse migratory PGCs have a low ability to adhere to gonadal tissue. Fibronectin does not seem to be implicated in short-term adhesion of mouse PGCs.

In conclusion, the method described in the present paper provides now a basis for the investigation of the biological significance and the biochemical characterization of the adhesive phenomenon between PGCs and follicular and Sertoli cells. In addition, the defined conditions of this *in vitro* system might allow study of a number of morphogenetic and interactive phenomena that occur during gonad development.

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