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Original Article

Immunohistochemical analysis of the segregation process of the quail germ cell lineage

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ABSTRACT An antiserum against quail 7 day gonadal germ cells was found to react specifically with gonadal germ cells of both sexes. Transverse sections from a range of early quail developmental stages were submitted to the antibody PAP reaction. Blastodiscs from the earliest uterine stages (II to X E.G & K) reacted very strongly, while the overall reaction gradually decreased in older blastoderms. At stage XIII both epiblast and hypoblast were weakly stained, but some large, PGC-like cells stained intensively. During gastrulation (PS formation) the reaction of the epiblast disppears quicker than that of the hypoblast. The newly formed mesoderm and entoderm do not react at all and the reaction gradually becomes limited mainly to the PGCs and somewhat to the primary hypoblast which is moving into the germinal crescent. The widely spread reaction at the early stages is thus gradually being restricted to the PGCs.

KEY WORDS: *germ cells, avian germ line, immunohistochemistry*

Introduction

Among the vertebrates, only in the anuran embryo has there been detected a germ plasm which is thought to contain the germ cell lineage determinants (Bounoure, 1934; Whitington and Dixon, 1975). No distinct germ plasm has ever been detected either in the zygote or in specific blastomeres of the other groups, and the mode and time of primordial germ cell (PGC) determination are still an enigma.

Studies on the origin of the PGCs and the germ cell lineage in avian embryos have been limited by the morphological homogeneity of early avian cell populations. The histochemical PAS staining for glycogen has so far been the only useful marker for differential labeling of the PGCs in early chick embryos (Meyer, 1959; Fujimoto *et al.*, 1976; Ginsburg and Eyal-Giladi, 1986, 1987). However, even this marker allows identification only at primitive streak (PS) stages and onwards. Indirect experiments have demonstrated the epiblastic origin of the PGCs (Eyal-Giladi *et al.*, 1981) and the existence of at least some determined PGCs already at stages XI-XII E.G&K (Sutasurja *et al.*, 1983; Ginsburg and Eyal-Giladi, 1986, 1987).

Recently, two monoclonal antibodies were found which recognize avian PGCs: the first one, QH1, was raised against 12-day quail bone marrow. It was found to bind to cells of the hemangioblastic lineage and also to recognize PGCs of the young quail blastoderm (Pardanaud *et al.*, 1987). A second antibody, EMA-1, which was raised originally against mouse embryonal carcinoma cells (Hahnel and Eddy, 1982, 1986), also recognized chick PGCs from the time of their detachment from the epiblast, and during their migration and colonization of the gonadal germinal epithelium (Urven *et al.*, 1988).

We have taken a direct approach to address the question of PGC lineage using antibodies that were raised against quail gonadal PGCs. These were used to analyze histologic sections of consecutive stages of quail embryos from early cleavage (uterine stages) to 7 days of incubation.

Results

Since the PGCs for immunization were obtained from day 7 quail embryonic gonads, we first studied the interaction of the immune serum (from consecutive bleedings – see Table 1) with embryonic tissues of the same stage. Positive reactions with the immune serum were consequently obtained from the fourth bleeding (B4) and on (B5,B6). The rabbit was sacrificed after B6,

Abbreviations used in this paper: E: epiblast; H, hypoblast; PGC, primordial germ cell; PS, primitive streak; En, entoderm; m, mesenchyme.

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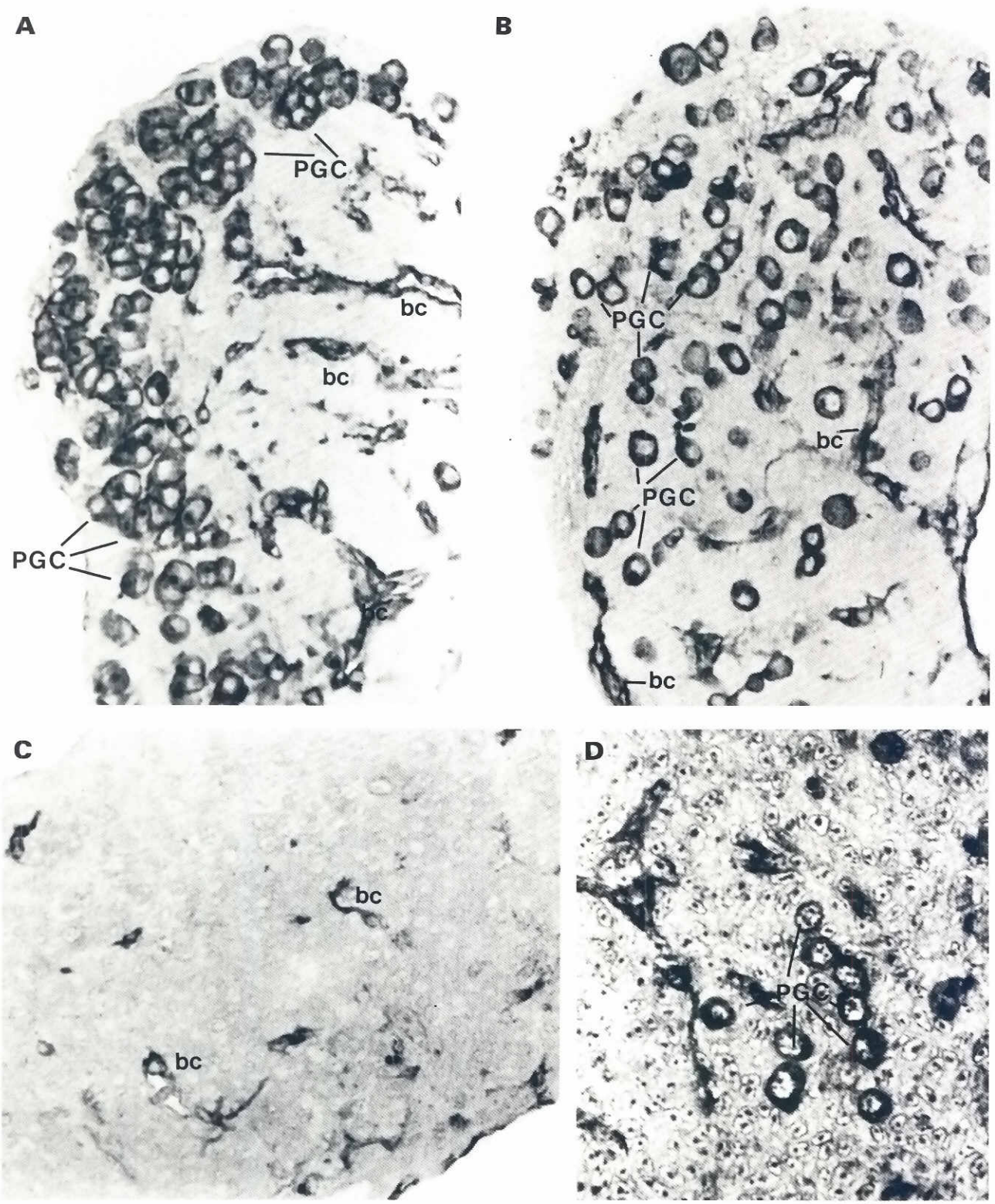

Fig. 1. Paraffin sections of 7 day quail embryonic gonads. x 480. (A) *PAP reaction of B4 1:100 on a section of female gonad.* **(B)** *PAP section of B4 1:100 on a section of male gonad.* **(C)** *PAP reaction of the preimmune Bo 1:100 on a section of male gonad.* **(D)** *A section of male gonad that was hematoxylin stained following the immunohistochemical reaction.*

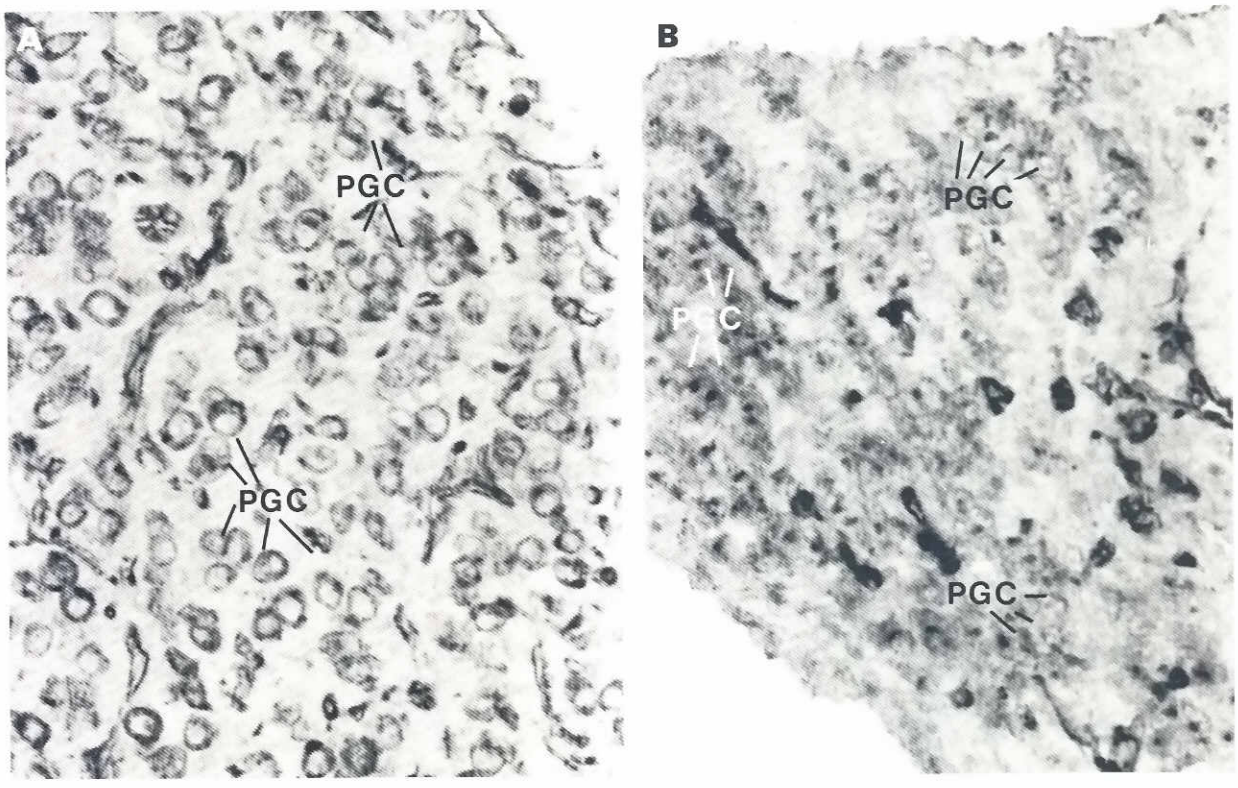

Fig. 2. Paraffin sections of 15 day quail embryonic gonads. x 480. (A) *PAP reaction of B4 1:100 on male gonad.* **(B)** *PAP reaction of B4 1:100 on female gonad.*

but throughout the present study only immune serum B4 was used.

Gonadal PGCs

Immune serum at dilutions of 1:100-1:500 reacted specifically with the cytoplasm of the undifferentiated germ cells of both sexes which populated the 7 day embryonic gonads. No reaction was detected in the mesodermal cells constituting the gonadal tissues (Fig. 1A, B, C). Other tissues taken from 7 day old quail embryos were also sectioned and tested with different dilutions of B4 antiserum. Limbs, heart and liver were negative. Yolk sac endoderm was the only tissue that reacted with the antiserum at dilutions of 1:100-1:500. The preimmune serum (Bo) was inactive against all target tissues tested. Both preimmune and immune sera reacted with capillary endothelial cells (Fig. 1). As both frozen and paraffin sections demonstrated comparable results we restricted all subsequent tests to paraffin sections, which were histologically much better preserved.

Quail gonadal PGCs were immunohistochemically reactive until at least 15 days of incubation, when the reaction was more prominent for male than for female PGCs (Fig. 2A, B). Older gonads were not checked. To check for species specificity, chick embryonic gonads were tested with the antiserum to quail PGCs. No reaction was detected in sections of 10 day old chick embryos, a stage comparable to 7 day old quail embryos.

Uterine stages (II, VII, X E.G&K)

All the cells in sections of these stages reacted very strongly and invariably with B4 antiserum at a dilution of 1:2000 (Fig. 3A). Preimmune serum at the same dilution gave entirely negative results (Fig. 3B).

Stage XIII (blastula)

The B4 immune serum reacted equally with both the epiblast and the hypoblast at dilutions up to 1:30,000. After absorption on liver cells from 7 day quail embryos (1 h, 4°C) the B4 immune serum was applied to the sections at a dilution of 1:1000. The absorbed serum distinguished some large, germ cell-like cells in the hypoblast that stained more intensely than their neighbors (Fig. 3C).

Gastrulating stages

During gastrulation the epiblast seemed to gradually lose its immunostainability with the B4 antiserum. In

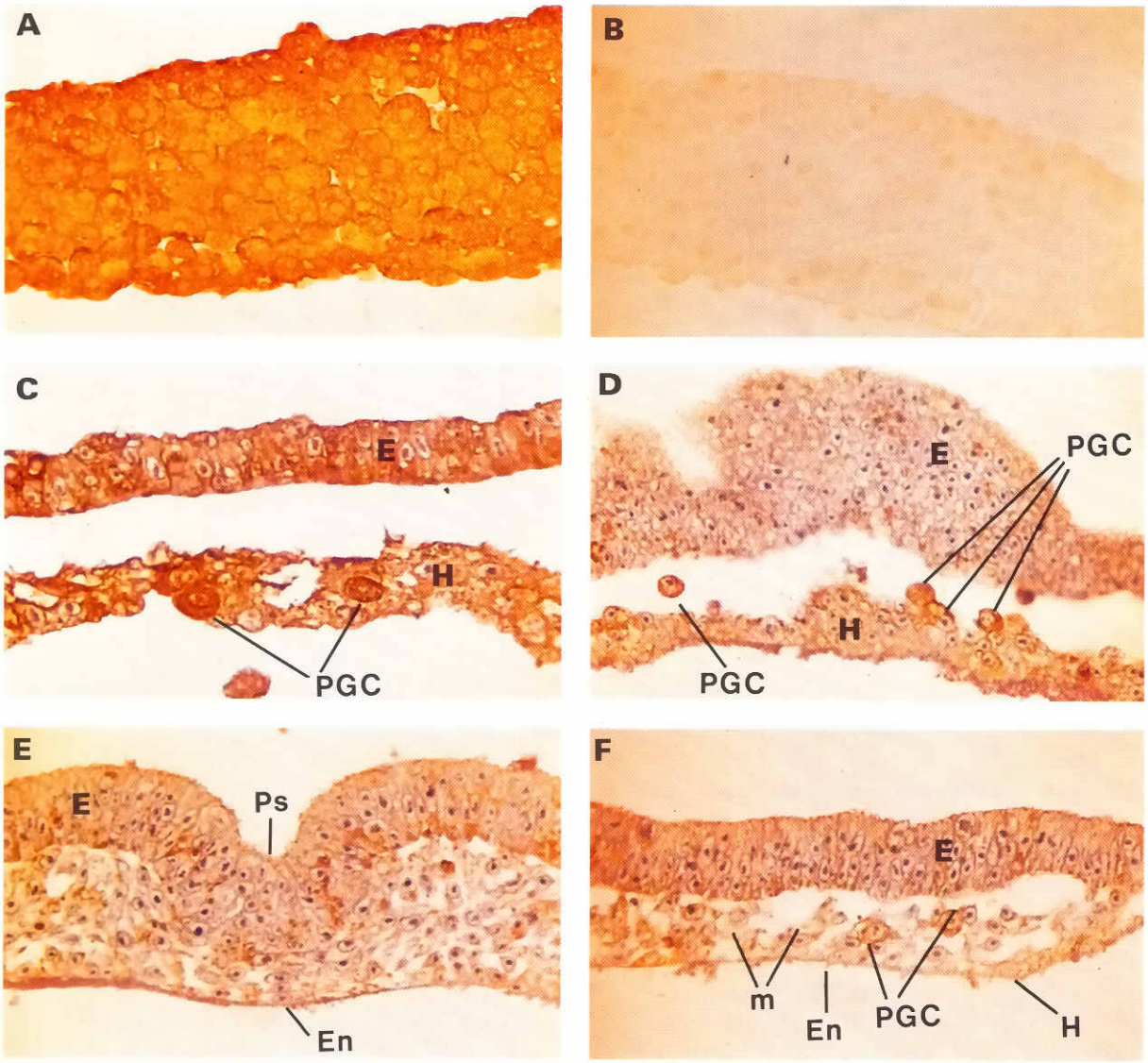

Fig. 3. Paraffin sections of quail at various developmental stages. x 300.(A) *PAP reaction of B4 antiserum 1:2000 on section of stage VII blastoderm.* **(B)** *PAP reaction of the preimmune Bo antiserum 1:2000 on section of stage VII blastoderm.* **(C)** *PAP reaction of B4 antiserum after absorption on liver cells, at dilution of 1:1000, on transverse section of stage XIII blastoderm, followed by hematoxylin staining.* **(D)** *PAP reaction of B4 antiserum 1:500 on transverse section made at the area of Hensen's node of stage 3 (H&H) blastoderm, followed by hematoxylin staining.* **(E)** *PAP reaction of B4 antiserum 1:500 at the area of the PS, posterior to D.* **(F)** *The same section as in E photographed lateral to the PS.*

the vicinity of the PS (Fig. 3E) several cells were detected in the epiblast which expressed a weak reaction mainly in their basal parts. The newly formed mesoderm did not react with the antiserum except for a few scattered cells situated lateral to the PS (Fig. 3F). Also, the definitive endoderm (EN) underneath the PS and lateral to it reacted negatively with the antiserum. In Hensen's node (HN) at the anterior end of the PS the reaction is very weak (Fig. 3D) and it entirely disappears from the epiblastic area anterior to the streak. However, underneath the non-reactive HN and more anterior to it, the lower layer stands out due to its positive reaction with B4 (1:500) antiserum (Fig. 3D). Similarly, several PGCs with an even more prominent positive reaction can be distinguished in the space between the epiblast and the hypoblast.

Discussion

The timing as well as the mechanism of the segregation of the germ line from the somatic cell lines in vertebrates is still unknown. With the exception of the anurans (Czolowska, 1972; Bounoure, 1934; Blackler, 1958, 1966; Whitington and Dixon, 1975; Ressom and

TABLE 1

SCHEDULE OF RABBIT BLEEDINGS AND IMMUNIZATIONS WITH PGCs

Days	Number of PGCs injected	Bleeding sample
1	16×10^4	Bo
14	8.4×10^4	B1
20	21.5×10^4	B2
36	20×10^4	B3
56	27×10^4	B4
76	20×10^4	B5
96		B6

Dixon, 1988), in oocytes or cleaving embryos of all other vertebrates no identifiable germ plasm was ever detected. Moreover it has been shown that in contrast to anurans, the PGCs of Urodeles (Sutasurja and Nieuwkoop, 1974), birds (Eyal-Giladi *et al.*, 1981) and mammals (Jacob, 1977; Mintz, 1971; Snow and Monk, 1983) are of epiblastic origin.

As to the mode of determination of the PGCs in the ectodermal-epiblastic layer, Sutasurja and Nieuwkoop (1974) claimed that in Urodeles the PGCs are induced in the ectodermal part of the animal hemisphere, by the ventral half of the vegetal hemisphere, implying that the animal cap is totipotent and capable of forming PGCs if exposed to the appropriate induction.

In mammals there is still a discussion as to whether the epiblast is composed of a totipotent cell population in which the somatic cell population is the one first determined by yet unknown restrictive events. This would leave a small population of unrestricted totipotent cells to become PGCs later on (McMahon *et al.*, 1983). Another approach favors the idea (Soriano and Jaenisch, 1986; Monk *et al.*, 1987) that it is the germ line which is first allocated. In birds, Ginsburg and Eyal-Giladi (1986, 1987) have demonstrated that most PGCs segregate from the central part of the epiblast. This leaves the question open whether the epiblast contains specific determinants that were allocated to it by a specific cytoplasmic section of the germinal disc, or whether the central area of the epiblast is exposed to inductive influences dictating the formation of the PGCs.

Several investigators have tried to tackle the dilemma of PGC origin and determination by tracing the developmental pathway of the germ cell line, using an immunological approach.

In the mouse, polyclonal antibodies were produced either against stem cells of teratocarcinoma (Artzt *et al.*, 1973; Gachelin *et al.*, 1976) or against germ cells (Heath, 1978). The results suggest that the germ cell lineage is derived directly from the embryonic ectoderm of the early implantation mouse embryo. However, the fate of the germ line at earlier stages remained unclear. A broader view regarding the early stages evolved from studies using a monoclonal antibody which was produced gainst a Forssman-like antigen present on the membranes of teratocarcinoma stem cells as well as on PGCs (Willison and Stern, 1978; Evans *et al.*, 1979). This antibody reacted positively with the entire inner cell mass in the early developmental stages. However, in somewhat later stages it stopped reacting with the ectoderm and was restricted only to the extraembryonic endoderm and the gonadal PGCs.

In Avians, the monoclonal antibody QH1 (Pardanaud *et al.*, 1987) shows an affinity to quail blastomeres, ranging between stages X E.G&K to 13 H&H, identified by the authors as PGCs. Unfortunately, it was not mentioned whether the above antibody, which is not specific to the germ line, was tested on earlier embryos. The same is true of the studies using the monoclonal antibody EMA-1 (Hahnel and Eddy, 1982, 1986) on chick cells (Urven *et al.*, 1988). This antibody reacted with some of the epiblastic cells at stages X-XI E.G&K and with some ingressing cells at stage XII. However, the identity of the reactive cells as PGCs could not be established at such early sages, due to the lack of morphological criteria. Only at stage XIV and onwards, could the positive EMA-1 cells be shown to be differentially PAS positive. The chick PGCs remained reactive to EMA-1 throughout the migration period and the colonization of the gonad. This study seems to support the notion of the epiblastic origin of the chick PGCs but does not contribute information as to their earlier origin. On the other hand, it demonstrates for the first time the existence of common antigens shared by the germ lineage of chicken and mouse.

Most of the studies mentioned above used polyclonal or monoclonal antisera raised against cells other than the germ line. Our approach, like that of Heath (1978), was to use an anti-PGC serum in order to try and detect a possible unique cytoplasmic antigen hopefully restricted to a specific region in the quail's germ which will prove to be connected to the germ line. What we found, however, was exactly the opposite, namely, a widespread reactivity of the early germ to the antiserum, which gradually became restricted to PGCs on the one hand, and to the primary hypoblast of the other. This result closely resembles the results with the Forssman-like antigen in the mouse, which reacts with the entire ICM of a $3^1/_2$-$4^1/_2$ day embryo, while later on the epiblast loses its reactivity, which remains strongly positive only in the endoderm and the PGCs.

It is worthwhile mentioning in this connection that the primary hypoblast of the quail's blastula (stage X E.G&K), which in our case reacted positively, continued to be positive also during the hypoblast's anteriorly directed movement onto the germinal crescent and the yolk sac.

Considering all the above information it seems that the different antigens which were shown to be connected to PGCs can be classified into two groups. One group of antigens was detected at post cleavage stages (Pardanaud *et al.*, 1987; Urven *et al.*, 1988) and their appearance seems to be correlated to already morphologically recognizable PGC differentiation. Some of these antigens may even be connected with cell migration or ingression. Other antigens, like the Forsmann-like antigen and the ones which react with our polyclonal antiserum, seem to be "pluripotent antigens" present at early cleavage, much before the appearance of identifiable PGCs. These antigens are initially uniformly dispersed. They gradually disappear from all the cells commited to become somatic, while they are retained in the PGCs and the primary hypoblast, which in birds is believed to be involved in the translocation of the PGCs into the germinal crescent (Ginsburg and Eyal-Giladi, 1986).

The results of the present study cannot be regarded as conclusive. However, since we used a polyclonal antoiserum raised against whole gonadal PGCs we stand a better chance of seeing our antiserum react with more than one antigen specific for PGCs.

All the evidence concerning the early stages both in mouse and quail indicates that during cleavage there is still no predetermined germ plasm and that all the pluripotent cells are potential PGCs. This agrees also with the conclusions of Sutasurja and Nieuwkoop (1974) concerning the Urodeles.

Our present data cannot distinguish between the two approaches mentioned above: 1) that the somatic cells are determined first, leaving a stock of pluripotent cells as PGCs; 2) that some active inductive influence is needed to preserve the pluripotency of the PGCs, which would otherwise be restricted to becoming somatic cells.

Materials and Methods

Immunization

For immunization, quail gonadal PGCs from embryos of 7 days of incubation were used. The isolated gonads of both sexes were cleaned of adhering tissues and then gently pressed between the bottom of a Petri dish and a coverslip (Heath, 1978) to the point of rupture. The cell suspension containing the fluid that was extruded from the gonads was filtered through a double layer of gauze and centrifuged for 10 min at 800xg. 90% of the pelleted cells were PGCs according to morphological criteria. The cells were immersed for 10 min in 1% formaldehyde, washed in PBS and injected intradermally in complete Freund's adjuvant into multiple sites of an adult female rabbit.

The protocol for immunization and the bleeding is shown in Table 1.

Preimmmune as well as immune sera were inactivated at 56°C for 30 min and stored at -20°C.

Immuno-peroxidase staining on sections

Frozen sections

Blastoderms and tissues were fixed for 10 min in 3% paraformaldehyde in PBS and washed in PBS. They were then incubated for 30 min in 0.1% glycine in PBS, whashed in PBS and then frozen in liquid nitrogen vapors. 6 µm-thick sections were cut by a kryostat and fixed on the slides for 5 min in acetone at -20°.

Paraffin sections

Blastoderms and tissues were fixed for 1 h in Brodski's fluid (1960) and then embedded in paraffin. 6 µm-thick sections were put on slides previously coated with gelatine.

Cellular antigens were visualized using the peroxidase anti-peroxidase technique, essentially described by Sternberger (1979). Some slides were also stained with hematoxylin following the immunohistochemical reaction. All the chemicals were purchased from Miles Yeda, Rehovot, Israel.

References

ARTZT, K., DUBOIS, P., BENNETT, D., CONDAMINE, H., BABINET, C. and JACOB, F. (1973). Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture. *Proc. Natl. Acad. Sci. USA 70*: 2988-2992.

BLACKLER, A.W. (1958). Contribution to the study of germ-cells in the Anura. *J. Embryol. Exp. Morphol. 6*: 491-503.

BLACKLER, A.W. (1966). Embryonic sex cells in Amphibia. *Adv. Reprod. Physiol. 1*: 9-22.

BOUNOURE, L. (1934). Recherches sur la lingee germinale chez la grenouille rousse aux premiers stades du developement. *Annls. Sci. Nat. (ser. 10) 17*: 67-278.

BRODSKI, V.Y. (1960). About techniques of fixation and tissue preparation for cytochemical and quantitative analysis. *Cytologia 3*: 605-613. (In Russian)

CZOLOWSKA, R. (1972). The fine structure of the "germinal plasm" in the eggs of *Xenopus laevis*. *Wilhelm Roux Arch. EntwMech. Org. 169*: 335-344.

EYAL-GILADI, H., GINSBURG, M. and FARBAROV, A. (1981). Avian primordial germ cells are of epiblastic origin. *J. Embryol. Exp. Morphol. 65*: 139-147.

EYAL-GILADI, H. and KOCHAV, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. *Dev. Biol. 49*: 321-337.

EVANS, M.J., LOVELL-BADGE, R.H., STERN, P.L. and STINNAKRE, M.G. (1979). Cell lineages of the mouse embryo and embryonal carcinoma cells; Forssman antigen distribution and patterns of protein synthesis. In *Cell Lineage, Stem Cells and Cell Detemination* (Ed. N. Le Douarin). INSERM Symposium No. 10, Elsevier.

FUJIMOTO, T., UKESHIMA, A. and KIYOFUJI, R. (1976). The origin, migration and morphology of the primoridial germ cell in the chick embryo. *Anat. Rec. 185*: 139-154.

GACHELIN, G., FELLOUS, M., GUENET, J.-L. and JACOB, F. (1976). Developmental expression of an early embryonic antigen common to mouse spermatozoa and cleavage embryos, and to human spermatozoa: its expression during spermatogenesis. *Dev. Biol. 50*: 310-320.

GINSBURG, M. and EYAL-GILADI, H. (1986). Temporal and spatial aspects of the gradual migration of primordial germ

cells from the epiblast into the germinal crescent in the avian embryo. *J. Embryol. Exp. Morphol. 95*: 53-71.
GINSBURG, M. and EYAL-GILADI, H. (1987). Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process. *Development 101*: 209-219.
HAHNEL, A.C. and EDDY, E.M. (1982). Three monoclonal antibodies against cell surface components on early mouse embryos. *J. Cell Biol. 95:* 156a.
HAHNEL, A.C. and EDDY, E.M. (1986). Cell surface markers of mouse primordial germ cells defined by monoclonal antobodies. *Gamete Res. 15*: 25-34.
HAMBURGER, V. and HAMILTON, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morph. 88:* 49-92.
HEATH, J.K. (1978). Characterization of a xenogeneic antiserum raised against the fetal germ cells of the mouse: cross-reactivity with embryonal carcinoma cells. *Cell 15*: 299-306.
JACOB, F. (1977). Mouse teratocarcinoma and embryonic antigens. *Immunol. Rev. 33*: 3-32.
McMAHON, A., FOSTEN, M. and MONK, M. (1983). X-chromosome inactivaction mosaicism in the three germ layers and the germ line of the mouse embryo. *J. Embryol. Exp. Morphol. 74*: 207-220.
MEYER, D.B. (1959). Application of the periodic acid Schiff technique to whole chick embryos. *Stain Technol. 35*: 83-89.
MINTZ, B. (1971). Clonal basis of mammalian differentiation. *Symp. Soc. Exp. Biol. 25*: 345-370.
MONK, M., BOUBELIK, M. and LEHNERT, S. (1987). Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development 99*: 371-382.
PARDANAUD, L., BUCK, C. and DIETERLEN-LIEVRE, F. (1987). Early germ cell segregation and distribution in the quail blastodisc. *Cell Differ. 22*: 47-60.
RESSOM, R.E. and DIXON, K.E. (1988). Relocation and reorganization of germ plasm in *Xenopus* embryos after fertilization. *Development 103*: 507-518.
SNOW, M.H.L. and MONK, M. (1983). Emergence and migration of mouse primordial germ cells. In *Current Problems in Germ Cell Differentiation* (Eds. A. McLaren and C.C. Wylie). Symposium of British Society for Developmental Biology, Cambridge University Press, pp. 115-135.
SORIANO, P. and JAENISCH, R. (1986). Retroviruses as probes for mammalian development: allocation of cells to the somatic and germ cell lineages. *Cell 46*: 19-29.
STERNBERGER, L.A. (1979). The unlabeled antibody peroxisidase-antiperoxidase (PAP) notochord. In *Immunochemistry* (2nd ed.). John Wiley & Sons, N. Y., pp. 104-169.
SUTASURJA, L.A. and NIEUWKOOP, P.D. (1974). The induction of the primordial germ cells in the urodeles. *Wilhelm Roux Arch. EntwMech. Org. 175*: 179-220.
SUTASURJA, L.A., YASUGI, S. and MIZUNO, T. (1983). Appearance of primordial germ cells in young chick blastoderms cultured *in vitro*. *Dev. Growth Differ. 25*: 517-521.
URVEN, L.E., ERICKSON, C.A., ABBOT, U.K. and McCARREY, J.R. (1988). Analysis of germ line development in the chick embryo using an anti-mouse EC cell antibody. *Development 103*: 299-304.
WHITINGTON, P.Mc.D. and DIXON K.E. (1975). Quantitative studies of germ plasm and germ cells during early embryogenesis of *Xenopus laevis*. *J. Embryol. Exp. Morphol. 33*: 57-74.
WILLINSON, K.R. and STERN, P.L. (1978). Expression of a Forssman antigenic specificity in the preimplantation mouse embryo. *Cell 14*: 785-793.

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