The role of the glycoconjugates in the migration of anuran amphibian germ cells

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SUMMARY
1. The presence of a large amount of glycoconjugates on the anuran amphibian germ cells was demonstrated using fluorescein isothiocyanate lectins binding specifically to D-galactose and at a lower level, by other lectins binding specifically to N-acetyl-galactosamine.
2. Glycoconjugates including D-galactose were found near the pseudopodial expansions and in the extracellular space, between germ cells and follicular cells. They were also disseminated in the cytoplasm.
3. The injection of PNA lectin (from Arachis hypogea) into the endoderm inhibited the migration of 90% of the germ cells. This inhibition was lectin-concentration dependent. Ultrastructural study of germ cells, the migration of which was inhibited, showed that they were degenerating. These results suggest that glycoconjugates are related to the migratory activity of germ cells.

RESUME
1. Les cellules germinales primordiales des amphibiens anoures sont caractérisées par l'abondance de glycoconjugués, révélés par des lectines fluorescentes toutes affines du D-galactose et à un moindre degré de la N-acetyl-galactosamine.
2. Les glycoconjugués porteurs de D-galactose sont présents au niveau des expansions pseudopodiales et dans l'espace extracellulaire séparant les cellules germinales des cellules folliculaires.
3. Les glycoconjugués sont en relation avec l'activité migratoire des cellules germinales. L'injection de lectine PNA (extraite de Arachis hypogea) entraîne une inhibition de la migration de 90% des cellules germinales. L'étude ultrastructurale des cellules germinales dont la migration est inhibée montre qu'elles subissent une lyse importante.

INTRODUCTION
It is possible by treating histological sections with lectins linked to fluorescein–isothiocyanate to show up lectin-binding sites and hence the glycosidic chains to which they are specifically bound. For example, Johnson & Smith (1976) have shown that lectins extracted from soybean (SBA) and from Canavalia ensiformis (Con A) bind to Xenopus laevis and Xenopus mulleri associated cells, and both Fargeix, Didier, Guillot & Damez (1980), and Didier, Fargeix & Didier (1980) have demonstrated the presence of glycosidic chains on the surface of avian germ cells.
Previous studies (Delbos, Saidi & Gipouloux, 1982), employing a variety of lectins which bind to different sugars, allow the conclusion that glycosidic residues on the surface of germ cells have functional importance. The time course of this labelling suggests that these glycoside residues are involved in migration. Similar studies with lectins like Con A, have an inhibitory effect on the morphogenesis of amphibian embryos since the addition, in vitro, of Con A to amphibian neural crest cells and to chick neural crest cells stops the migration of these cells (Moran, 1974a, b; Boucaut, Bernard & Aubery, 1977; Boucaut, 1978).

In order to know if the migratory activity of germ cells is perturbed in vivo by the action of lectins possessing a particular affinity for these cells, we injected these lectins into the endoderm of early embryos during the migration of germ cells. The results were observed by optical microscopy and by transmission electron microscopy.

**MATERIALS AND METHODS**

Experiments were carried out on *Rana dalmatina* embryos, between stages 26 and 30 (Cambar & Marrot, 1954), on *Bufo bufo*, between stages III₅ and III₁₀ (Cambar & Gipouloux, 1956), on *Xenopus laevis* between stages 30 and 47 (Nieuwkoop & Faber, 1956).

Fluorescein–isothiocyanate lectins (FITC) were used for the cytochemical detection of membrane glycoconjugates. The lectins used were extracted from *Arachis hypogea* (PNA), from soybean (SBA), from *Phaseolus vulgaris* (PHA), from *Phytolacca americana* (PWM), from *Lens culinaris* (LCA), from *Triticum vulgaris* (WGA) and *Ulex europaeus* (UEAF). They were provided by Sigma.

The samples were fixed in Bouin-Hollande solution for 2 h, dehydrated, then embedded in paraffin. The sections were 5 µm thick. Sections were treated with fluorescent lectins according to the Avrameas technique (Avrameas, 1969). After rehydration sections were incubated at 25 °C with lectins at a concentration of 500 µg/ml in 0.01 M-PBS (pH 6.8). For control animals, incubation was made in 0.01 M-PBS alone (pH 6.8). The specificity of each lectin was checked by carrying out an incubation with the lectin plus its inhibitory sugar. The observation was made with a Zeiss microscope fitted with an excitation filter BG 12.

In order to visualize the sugars of the surface and the intracellular sugars, glycosylated ferritin was used and sections observed with electron microscope. The samples were fixed with 6% glutaraldehyde in cacodylate buffer 0·1 M (pH 7.4) for 1 h. Sections were treated with PNA solution (250 µg/ml) or SBA (250 µg/ml) in 0·1 M-cacodylate buffer (pH 7·4) for 2 h. The samples were transferred to buffer, then incubated in a lactosyl-ferritin solution at a concentration of 250 µg/ml in PBS for 3 h. Some samples were treated with lectin and 0·2 M-lactose together, in order to prevent the reaction. Then, they were post-fixed in 2% osmic acid in cacodylate buffer for 1 h at 0°C. They were dehydrated and embedded in Epon. Sections were observed without staining.
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The other embryos were prepared for ultrastructural study: fixation involved 1 h in 6% glutaraldehyde, 1 h in 2% osmic acid. The specimens were embedded in Epon. Sections were stained by uranyl acetate and lead citrate and observed with a Philips 201 electron microscope.

The lectin injections (15 μl, about 7 μg lectin per embryo) were made, each day into the endodermal mass of the embryos with a micropipette during the time of the migration of germ cells, according to the following scheme.

LOT I : 100 embryos, injection of PNA each 24 h
LOT II : 100 embryos, injection of PNA each 48 h
LOT III : 100 embryos, injections of PNA each 48 h, then each 24 h after 3 days
LOT IV : 100 embryos, injection of SBA each 24 h
LOT V : 100 embryos, injection of PBS each 24 h
LOT VI : 100 embryos, injection of PNA + galactose each 24 h
LOT VII : 100 embryos, no injection (control animals)

At the stage during which germ-cell migration was complete in control animals, one part of the embryos was fixed with Bouin liquid and embedded with paraffin wax. Sections were stained with Groat haematoxylin.

RESULTS

I. Presence of glycoconjugates on the germ cells

Selective labelling of anuran germ cells is obtained with PNA and SBA which both bind to galactose and to a lesser degree, with PHA which binds to galactosamine (Figs 1, 2, 3, 4). The lectins extracted from *Ulex* (UEAF binding to fucose), from *Triticum* (WGA binding to N-acetyl-galactosamine) and from *Lens* (LCA binding to mannose and glucose) do not show a specific reaction with germ cells (Figs 5, 6).

The ultrastructural study gives some information on the precise localization of specific carbohydrates on germ cells. The observations were made on *Xenopus laevis* embryos (stage 47) and by comparison, on *Rana dalmatina* embryos (stage 39), at stages during which fluorescence is the most intensive. The use of the double staining with PNA lectin and lactosyl-ferritin reveals the presence of many spherical granules of 9–15 nm diameter (about the diameter of ferritin granules) (Fig. 7). These granules are distributed regularly on the pseudopodia of the germ cells, collected in clusters (Figs 8, 9) in the extracellular space separating germ cells from adjacent follicular cells, but also distributed rather uniformly in the cytoplasm of the germ cells (Fig. 12).

In *Xenopus* embryos treated by lactosyl-ferritin without lectin, or by PNA with galactose or lactose, we do not observe ferritin granule deposits. Epidermal cells or pronephric duct cells of the embryos treated at the same time by PNA and lactosyl-ferritin, as control cells, show no specific labelling (Figs 10, 11).
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II. Injection of PNA

Counting of germ cells in the genital ridges shows notable differences between control and treated animals. In embryos treated by PNA injections, the number of germ cells reaching genital ridges is very low. Genital ridges are practically devoid of germ cells (Fig. 13). Ectopic germ cells are observed easily in the intestine part (Figs 15, 16). Numbers of germ cells were obtained by counting their nuclei, which have a characteristic shape and coloration.

We observed the same number of germ cells in untreated gonads or those treated by regular injections of PBS (or PNA with galactose) – control animals (Fig. 14). Counting of germ cells in the different classes of treated animals gave the following results (Table I).

Examination of germ cells staying in the endoderm reveals that these cells are degenerating.

DISCUSSION

Using some fluorescent lectins, we have demonstrated the particular abundance of certain glycoconjugates on germ cells. The most intense fluorescence was obtained with PNA which binds to galactose and to a lesser degree with SBA and PHA which both bind to N-acetyl-galactosamine.

The importance of glycoproteins in the migration of germ cells has previously been suggested by many authors (Cuminge & Dubois, 1974; Moran, 1974b; Lee, Karasanyi & Nagele, 1978). Thus, anuran germ cells seem to be characterized by the presence of a large amount of galactose and N-acetyl-galactosamine. However, the observed fluorescence seemed to be located in both cytoplasm and cell membrane.

We have tried to localize these glycosidic residues by electron microscopy. The great specificity of lectins for carbohydrates has been used for ultrastructural

Fig. 1. Intragonadal primordial germ cells after incubation with FITC-labelled PNA and galactose: no fluorescence is observed (×500). pgc, primordial germ cell; pd, pronephric duct. (*Rana dalmatina, st. 39*).

Fig. 2. Intragonadal primordial germ cells after incubation with FITC-labelled PNA (×500). (*Rana dalmatina, st. 39*). pgc, primordial germ cells; pd, pronephric duct.

Fig. 3. Intragonadal primordial germ cells after incubation with FITC-labelled PNA (×1200). (*Bufo, IIIio*). pgc, primordial germ cell; pd, pronephric duct.

Fig. 4. Intragonadal primordial germ cell after incubation with PBS alone (Control animals). No fluorescence is observed (×500). pgc, primordial germ cell; pd, pronephric duct.

Fig. 5. Intragonadal primordial germ cells after incubation with FITC-labelled UEAF (×500). pgc, primordial germ cell; pd, pronephric duct.

Fig. 6. Intragonadal germ cell after incubation with FITC-labelled LCA (×500). pgc, primordial germ cell; pd, pronephric duct.
detection of membrane and intracellular glycosidic sites (Nicolson, 1974, 1976, 1978). Lectins are not electron opaque but have the advantage of possessing at least two attachment sites for sugars. Thus, it is possible to attach them to electron-dense molecules like ferritin.
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We observed that the ferritin granules bound to the receptor sites of PNA were situated in the cytoplasm of germ cells and on their cellular surface. This fact confirms our observations using fluorescence microscopy. Furthermore, we observed a uniform distribution of ferritin granules on the pseudopodial expansions of germ cells and in the extracellular spaces separating germ cells from adjacent follicular cells. The absence of ferritin deposits after treatment by PNA and galactose confirms the specificity of the reaction.

Previous work has demonstrated (Delbos, Guennoun & Gipouloux, 1981) the existence of an abundant extracellular matrix particularly near the pseudopodial expansions. These results suggest that the extracellular matrix, which comprises collagen fibres and glycosaminoglycans associated with glycoconjugates of which

Fig. 7. Presence of ferritin granules (arrow) in extracellular space and in the cytoplasm of primordial germ cell (×80,000). pgc, primordial germ cell; fc, follicular cell.

Fig. 8. Presence of ferritin granules (arrow) in clusters near the membranes of the germ cells (×51,000). pgc, primordial germ cell.

Fig. 9. Same aspect of ferritin granules (arrow) (×78,000).

Fig. 10. Aspect of the epidermal cells in embryos treated by lactosyl-ferritin. No deposits of ferritin granules (×40,000).

Fig. 11. Aspect of the pronephric duct cells in embryos after the same treatment (×40,000).
Fig. 13. No germ cell is observed in genital ridges of embryos treated by PNA injections (×300). gr, genital ridge; ip, intestinal part.

Fig. 14. Germ cells are observed in genital ridges of embryos treated by injections of PBS or PNA with galactose (×300). gr, genital ridge; ip, intestinal part.

Fig. 15. Aspect of the germ cells observed in intestinal part (arrow) of embryos treated by PNA injections (×120).

Fig. 16. Germ cells observed in intestinal part (arrow) (×300).

Table 1. PGC number

<table>
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<tr>
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<th>Intragonadal PGC</th>
<th>Intraendodermal PGC</th>
<th>Total</th>
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<td>Controls</td>
<td>20 ± 0.57</td>
<td>—</td>
<td>20 ± 0.57</td>
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<tr>
<td>Treated with PNA 24 h</td>
<td>3.72 ± 0.54</td>
<td>12.0 ± 1.32</td>
<td>15.72 ± 1.12</td>
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<tr>
<td>PNA 48/24 h</td>
<td>5.20 ± 2.60</td>
<td>7.80 ± 3.17</td>
<td>13.0 ± 5.20</td>
</tr>
<tr>
<td>PNA 48 h</td>
<td>5.33 ± 3.17</td>
<td>5.33 ± 1.32</td>
<td>10.66 ± 4.41</td>
</tr>
<tr>
<td>SBA 24 h</td>
<td>9.20 ± 1.24</td>
<td>5.22 ± 2.18</td>
<td>14.42 ± 3.39</td>
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Fig. 17. Percentage of gonadal primordial germ cells in treated embryos compared with untreated ones. 1: PNA (24 h); 2: PNA 48/24 h; 3: PNA 48 h; 4: SBA 24 h; 5: untreated embryos (physiological liquid).

The most abundant would be D-galactose, constitutes an available substrate for the movement of cells in migration (Lee et al. 1978). In addition, D-galactose could be an energy source together with glycogen for the active migration of germ cells.

After injection of PNA into the endodermal mass of embryos, the total number of germ cells observed in embryos treated by SBA and PNA is always lower than that observed in controls. There are two possible reasons for this: on one hand, the difficulty of identifying intraendodermal germ cells, and on the other the degeneration of a great number of germ cells which remained 'endodermal'. A statistical study reveals that differences between populations of intragonadal germ cells of control animals and of embryos treated by injections of PNA each day, are greatly significant. Fig. 17 gives the percentage of intragonadal germ cells observed in the different classes of treated embryos in comparison with the number of germ cells observed in controls. Furthermore, ultrastructural observation of intraendodermal germ cells reveals that these cells are degenerating.

The effect of lectins on migration seems clear since inhibition of migration is
lectin-concentration dependent. Furthermore, a combination of galactose and PNA breaks the inhibition produced by lectin alone. The observations reported in this present paper suggest that the inhibitory action of PNA on germ cell migration is produced either on the germ cell membrane or on the extracellular matrix adjoining germ cells, masking the galactose residues of the glycoconjugates which play a role in migration. It is possible that PNA (with two receptor sites of galactose) associates simultaneously with membrane galactosyl residues of germ cells, and with those of the extracellular matrix, preventing their migration.

In summary, the observations reported are consistent with an important role of glycoconjugates in the migration of amphibian germ cells.

REFERENCES


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