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Relationship between lectin-affinity granules in anuran embryos and formation of primordial germ cells

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Summary. Embryos of the anuran, *Rana nigromaculata*, contained granules with a specific affinity for Con A and GS-I. Larvae derived from embryos injected with these lectins had a noticeable reduction in both the number and size of primordial germ cells (PGCs). This observation suggests that the granules are somehow involved in the formation of PGCs.
Key words. Lectins; granules; anuran embryos; primordial germ cells.

In anuran embryos there is a specific portion (germ plasm) of the cytoplasm of some cells that contributes to the formation of primordial germ cells (PGCs), as conclusively demonstrated by Blackler [1], who confirmed the initial observation by Bounoure [2]. However, it is not possible to detect the germ plasm, or cells that contain this cytoplasm, throughout the duration of embryogenesis in all anuran species. In some species, the cells with germ plasm (presumptive PGCs = pPGCs) can barely be seen in embryos from the neurula to the hatching stage. *Rana nigromaculata*, the Japanese pond frog, is one such species [3]. Recently, I reported that a certain fraction of granules derived from embryos at the tail-bud stage contributes significantly to the proliferation of PGCs [4]. However, the particular granules that play a role in the proliferation of PGCs have not been characterized. If it were possible to purify the effective granules, they would be very helpful in our attempts to understand the mechanism of formation of PGCs.

In the present study, lectins are used to identify specific granules in sectioned preparations. Such granules might correspond to the PAS-positive granules (granules stainable with periodic acid-Schiff stain) that are found in specific locations during embryogenesis of some species in which pPGCs are not detectable [3]. PAS-positive granules appear to be a valid candidate for such a function, as judged from their size and behavior. The effects of lectins on the formation of PGCs were then examined. Based on the results, a discussion is presented as to whether lectins are available that might help in the purification of granules related to the formation of PGCs.

Materials and methods

Eggs of the Japanese pond frog, *Rana nigromaculata*, were artificially inseminated by routine methods in our laboratory. Room temperature was not specifically regulated (15–22 °C). Two experiments were performed: the detection of materials with an affinity for lectins; and the effects of lectins on the formation of PGCs.

In the first experiment, embryos at the tail-bud stage were fixed in cold (– 20 °C) Gendre's solution for two days and dehydrated in absolute ethanol (changed three times a week) at the same temperature to prevent the embryos from skrinking. These samples were cut into 6-μm serial paraplast sections, which were then reacted with ten lectins, each conjugated with rhodamine: BPA (*Bauhinia purpurea* agglutinin), Con A (*Concanavalia ensiformis*, jack bean, agglutinin), DBA (*Dolichos biflorus*, horse gram, agglutinin), GS-I (*Griffonia simplicifolia* I agglutinin), GS-II (*Griffonia simplicifolia* II agglutinin), MPA (*Maclura pomifera*, osage orange, agglutinin), PNA (*Arachis hypogaea*, peanut, agglutinin), SBA (*Glycine max*, soy bean, agglutinin), UEA (*Ulex europaeus*, grose, agglutinin) and WGA (*Triticum vulgaris*, wheat germ, agglutinin) (EY Laboratory, Inc., San Mateo, CA). Each lectin was used at a concentration of 0.2 mg/ml in a suitable buffer as indicated by the supplier. The sections were incubated with lectin for 30 min at 25 °C.

The preparations were observed by fluorescence microscopy.

In the second experiment, non-conjugated lectins were injected into the middle abdomen of embryos at the neural-tube stage, when the PAS-positive granules appeared to locate at the far ventral side of the archenteron (mid-gut). Each embryo was injected with 1 µl of a solution of 1 mg/ml of lectin in buffer [in general, 0.01 M phosphate buffer solution (PBS)] with a glass micropipette (ca 100 µm in diameter at the top). The embryos were fixed in Bouin's solution for 20 h at room temperature, when each had completely formed an operculum. These samples were then cut into 10-µm serial paraplast sections and stained with hematoxylin-eosin.

Results

Affinity of lectins for cellular components. In this experiment, there were no lectins with characteristic affinity for regions around nuclei or in any other regions of cells in embryos at the tail-bud stage. These results differ from those for migrating PGCs or pPGCs reported by Delbos et al.[5, 6].

The observations suggested that the lectins could be divided roughly into three types. One type of lectin did not seem to have any characteristic affinity for any components of the yolk cells around and at the ventral side of the mid-gut. Such lectins were DBA, GS-II, MPA and PNA. The second type of lectin had a specific affinity for a few granules in or among some cells. The fluorescence of the granules was intense and nearly uniform. The granules appeared mostly in or among yolk cells near or at the ventral side of the mid-gut, and rarely in other regions (e.g., in the somite or epithelial layer). The granules were about 3 µm in diameter. Lectins of this type were UEA, BPA and WGA.

The third type of lectin is of considerable interest here because these lectins had a specific affinity for numerous granules. These granules were mostly situated around and at the ventral side of the mid-gut. This type of lectin included the three lectins Con A, GS-I and SBA. With SBA, two kinds of granules were detected: granules similar to those mentioned above and larger ones (about 5 µm in diameter). In the case of Con A and GS-I, small granules (1 µm) were labeled in addition to the above-mentioned two kinds (fig. 1 B and C). Although some granules were collected within a few cells around the mid-gut, most of them appeared among or on yolk cells at the ventral side of the gut. In the case of SBA and Con A, the fluorescence on granules was intense and almost uniform. In the case of GS-I, the fluorescence on the granules was ring-like, differing from that seen with the other lectins. In addition, the shapes and distribution of the granules closely resembled those of PAS-positive granules (fig. 1 A).

Effect of lectins on the formation of PGCs. The table shows a summary of the results observed, in terms of the numbers and sizes of PGCs. The results are listed for two lectins of each type mentioned above. Fifteen embryos in each case were treated with PBS or with lectin, except in the case of Con A, where twenty embryos were used. Data from some embryos in each group are omitted from the table because they failed to develop normally. The control group consisted of larvae derived from embryos injected with PBS. The result was fundamentally similar to that obtained with animals without any injection (data not shown) in terms of the number and size of PGCs. The diameter of each PGC was measured along its major axis, as far as possible, in ten animals of each group.

Larvae in PNA- and MPA-treated groups had similar numbers of PGCs (35 and 34 on average, respectively) to

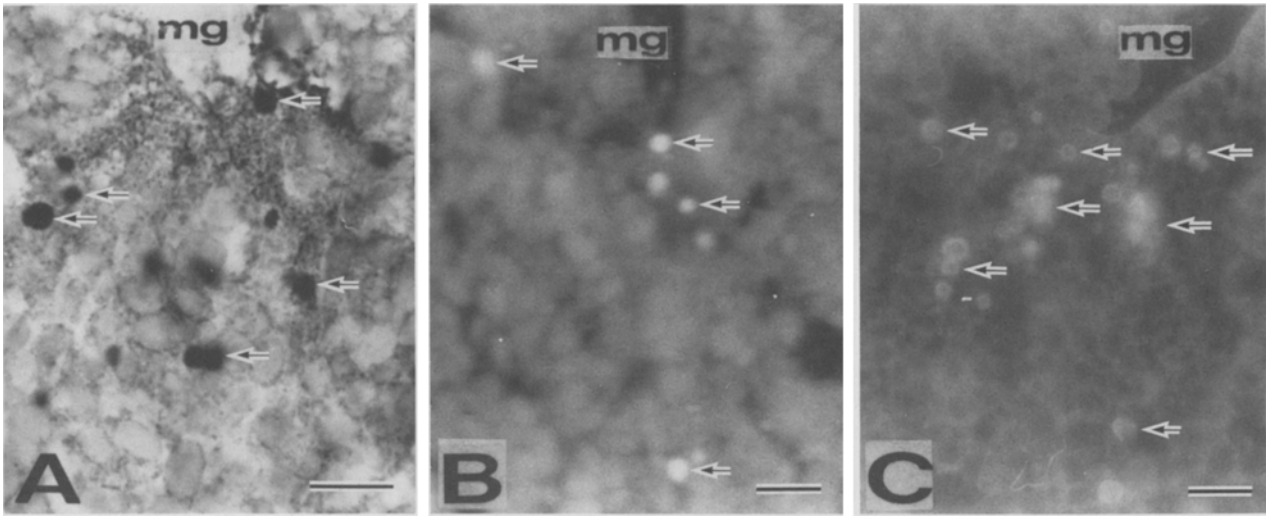

Figure 1. Cross-sections of embryos, about a third of the way from the anus, at the tail-bud stage. Bar, 10 µm. *A* Periodate-Schiff (PAS) reaction. *B* Con A conjugated to rhodamine. *C* GS-I conjugated to rhodamine. mg, mid-gut; Arrows indicate granules that reacted with PAS or coupled with lectins.

The average number and diameter (μm) of primordial germ cells (PGCs) in larvae derived from embryos treated with various lectins.

Group	Affinity* for lectin	No. of animals	Mean no. of PGCs ± SD	Min.–Max.	No. of PGCs**	Mean diameter of PGCs ± SD
Control		14	36 ± 4	28–46	328	23.8 ± 0.4
MPA	–	11	34 ± 3	28–39	316	24.0 ± 0.7
PNA	–	13	35 ± 5	24–44	315	24.4 ± 0.8
UEA	+	12	34 ± 5	22–41	339	21.0 ± 0.7
WGA	+	12	33 ± 6	17–41	320	20.3 ± 1.2
CON A	++	17	27 ± 7	16–37	245	18.9 ± 0.9
GS-I	++	11	28 ± 11	5–42	259	18.9 ± 1.4

*Affinity of lectin for specific granules. **Total number of PGCs measured in ten larvae. (MPA, treated with *Maclura pomifera* agglutinin; PNA, Peanut agglutinin; UEA, *Ulex europaeus* agglutinin; WGA, Wheat germ agglutinin; Con A, *Concanavalia ensiformis* agglutinin; GS-I, *Griffonia simplicifolia* agglutinin.)

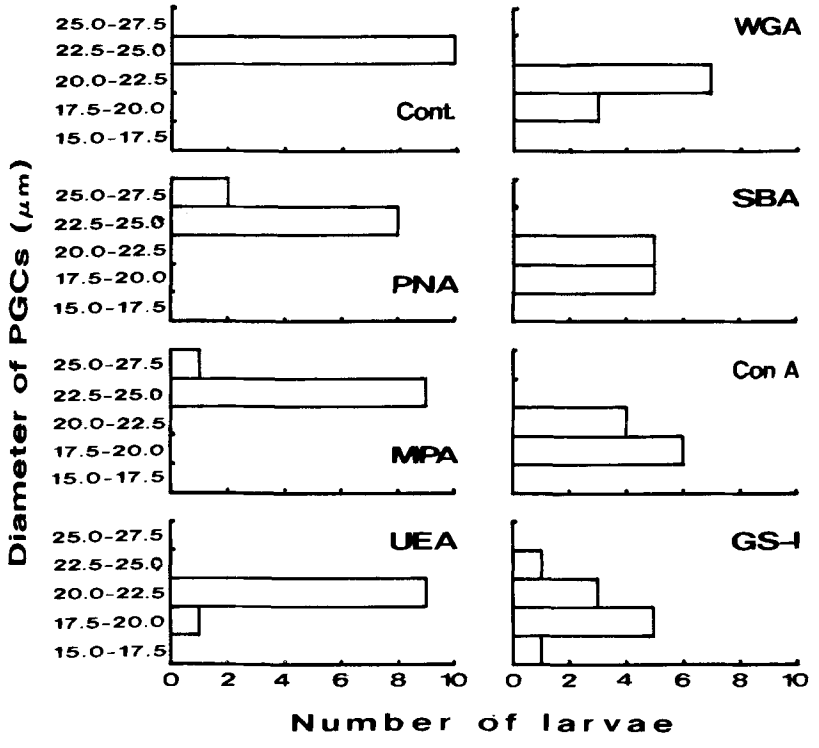

Figure 2. Distribution of animals derived from embryos injected with PBS or lectins in terms of the mean diameters of primordial germ cells (PGCs) grouped at intervals of 2.5 μm.

that (36) in the control. The diameters of PGCs in these larvae (24.4 and 24.0 μm on average, respectively) differed little from that (23.8 μm) in the control. In UEA- and WGA-treated groups, larvae showed a tendency towards a slight reduction in the number of PGCs (34 and 33 on average, respectively). Furthermore, the PGCs in these larvae had mostly lost many yolk platelets and were distinctly smaller in size (21.0 and 20.3 μm on average, respectively). In embryos injected with Con A or with GS-I, both the number (27 and 28, respectively) and size (18.9 μm) of PGCs were noticeably reduced, and the deviation from control values was statistically significant (t-test, $p < 0.01$). A few of these larvae had an extremely small number of PGCs (see table). Many of the PGCs had completely lost their yolk platelets. Figure 2 shows the distribution of animals in terms of the average diameter of PGCs, tabulated at intervals of 2.5 μm. The distribution of animals shifted clearly to those with PGCs of smaller diameter after injection of Con A or of GS-I. In the case of GS-I, although one animal had larger PGCs on average than those of the others, this animal had only five PGCs.

Discussion

In considering the present observations, it should first be noted that there was no evidence of specific affinity for PNA in embryos of *R. nigromaculata* at the tail-bud stage, although this lectin has a specific affinity for PGCs

and pPGCs in some anuran species[5-7]. In contrast, SBA, Con A and GS-I had a high affinity for granules in specific regions. These granules were distributed widely on the ventral side of the mid-gut and appeared on or among yolk cells, while some appeared to collect in a few cells around the mid-gut. Their shape and distribution closely resembled those of PAS-positive granules in the embryos of this species and of *R. brevipoda* at the same stage[3]. Therefore, these lectin-affinity granules may migrate separately from cells at the far ventral side of the mid-gut toward cells on the dorsal side, in view the behavior of PAS-positive granules during embryogenesis. This possibility is discussed below.

The second question concerns a possible correlation between the effects of lectins and the number of PGCs. The lectins with no affinity for granules had no effect on the formation of PGCs. In contrast, the other lectins had a more or less inhibitory effect. In particular, Con A and GS-I were very effective, in parallel to their high affinity for the specific granules. A similar effect of lectins was demonstrated in another experiment (injection of embryos with a fraction of granules prepared by centrifugation): granules pre-treated with GS-I did not contribute at all to the proliferation of PGCs, unlike the granules pre-treated with PNA or MPA (unpublished data).

The reduction in the size of PGCs in experimental animals was also of interest, since such a phenomenon has been reported in anuran larvae derived from UV-irradiated or overripe eggs[8-10]. In the latter, it was suggested that the alterations in PGCs were the result of the inhibitory migration of germ plasm during cleavage stages. Therefore, the present results suggest that the alterations in PGCs, in terms both of number and of size, may have resulted from an impediment in the normal migration of functional granules due to the lectins. If such is the case, it is possible that the lectin-affinity granules include some determinant of germ cells and are directly involved in the formation of PGCs. In other words, it is likely in this species, that the determinants leave the cells in which they remained during the blastula stage and move separately toward a new position, through yolk cells, through the use of an appropriate antigen on their envelopes. Given the results obtained from the PNA-treated group, it is probable, in *R. nigromaculata*, that the PGCs are not determined at earlier embryonic stages, such as the blastula stage. This suggestion is in contrast to the general assumption about the determination of PGCs in anura. It may be difficult to purify the target granules from other granules because many lectins have some affinity for yolk platelets. In particular, Con A, SBA and WGA had considerable affinity for yolk platelets, although it is uncertain whether they have the same affinity for small yolk platelets that have been digested to a large extent. If the lectins have only weak affinity for small yolk platelets, lectin-coated beads should facilitate isolation of the target granules, since large yolk platelets are not included in the granular fraction obtained by centrifugation (crude mitochondria).

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Involvement of a sperm aminopeptidase in fertilization of the sea urchin

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Summary. Inhibitory efficiencies of bestatin methyl ester and its nine analogs for sea urchin sperm aminopeptidase activity were similar to the efficiency of the same compounds as inhibitors either of sperm respiration or of fertilization. This suggests that a sperm aminopeptidase plays a role in fertilization in the sea urchin, possibly through its involvement in sperm respiration.
Key words. Aminopeptidase; bestatin; fertilization; respiration; sperm; sea urchin.

Some proteases of spermatozoa and eggs are thought to be involved in fertilization in echinoderms. Several lines of evidence have indicated that a chymotrypsin-like enzyme of the sperm is a lytic agent[3,4] (lysin), which allows the sperm to penetrate through the vitelline coat of the eggs, and that a trypsin-like enzyme of the eggs partici-