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Study of the Growth Regulation of Preimplantation Mouse Embryos Using Concanavalin A[1] (37832)

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(Introduced by H. Koprowski)
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Information on the growth regulation of preimplantation mammalian embryos is limited and has been concerned exclusively with changes in DNA, RNA, and protein synthesis (1–4). The involvement of cell membrane in the growth regulation of somatic cells was clearly demonstrated using plant lectins (5, 6). Cells of early mammalian embryos grow exponentially (7), and in this aspect they resemble tumor and/or virus-transformed somatic cells. Since lectins agglutinate tumor and transformed somatic cells and also change their growth pattern (5, 6), we have decided to study the cell membrane properties and growth pattern of the early mouse embryo after exposure to the lectin, Concanavalin A (Con A).

Materials and Methods. The preparation and culture of unfertilized eggs and embryos. Ovulation in ICR Swiss albino mice was stimulated by injections, 48 hr apart, of pregnant mare serum (PMS) and human chorionicgonadotropin (HCG); unfertilized eggs were obtained from oviducts 14–16 hr after HCG injection. Cumulus oophorus was removed from the eggs by hyaluronidase (Wydase, Wyeth Labs, Inc., 50 IU/ml) treatment. To obtain vitelluses, i.e., unfertilized eggs without zona pellucida, the eggs were exposed for 3 min at 37° to pronase (Calbiochem, Lot 101185) at a concentration of 2 mg/ml diluted in Whitten medium (8) with 1% polyvinylpyrrolidone. Parthenogenetical stimulation of unfertilized eggs was done according to the method described by Graham (9). The embryos were obtained from the same strain of mice by flushing them from oviducts at the appropriate time after mating. When the vaginal plug was found during morning inspection, insemination was considered to have occurred at 1 AM and the age of the embryo calculated accordingly. Zonae pellucida of 1-celled embryos (16 hr old), 2- and 4-celled embryos (46–48 hr old), and 6–8-celled embryos (60–64 hr old) was removed by pronase as above. The embryos were cultivated in Falcon dishes filled with Whitten medium supplemented with 0.3% BSA (Sigma, Lot 22C-8050) at 37° in a CO_2 incubator.

Binding of ^{125}I-Con A. One-hundred vitelluses and one-hundred embryos at each of different stages of development were processed together. After removal of the zona pellucida and washing in Whitten medium, they were incubated 30 min at 37° with 50 μg/ml of ^{125}I-Con A (sp act 2×10^5 cpm/μg Con A). ^{125}I-Con A was prepared by chloramine T method of iodination and purified by binding to a Sephadex G-50 column. The dose of ^{125}I-Con A was chosen on the basis of the dose relationship of Con A binding to somatic cells. In one set of experiments, vitelluses were incubated for 30 min with 50 μg/ml of ^{125}I-Con A in the presence of 0.05 *M* methyl-α-D mannopyranoside (MAM) to estimate the nonspecific binding of Con A. After binding, the cells were extensively washed, and the radioactiv-

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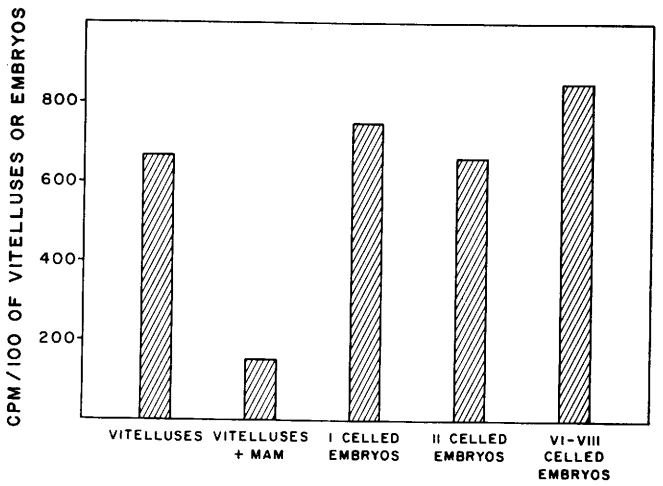

FIG. 1. Amount of ^{125}I-labeled Con A bound to vitelluses and embryos in different stages of development.

ity of the samples was determined with a gamma spectrometer. The radioactivity of the sample of vitelluses incubated with labeled Con A in the presence of MAM was considered as background during calculations of the binding of Con A to the cells.

Agglutination by Con A. Vitelluses and the zona-free embryos were prepared as described above. Cells (20–30/sample) were incubated for 15 min at room temperature with Con A at concentrations ranging from 1 to 2,000 μg/ml. The cells were then aspirated into a micropipet in order to increase intercellular contact.

The effect of Con A on growth of embryos. Two-celled (46–48 hr old) and 4-celled embryos (46–48 hr old) without zona pellucida were cultivated in Falcon dishes in Whitten medium containing 0.3% BSA in the presence of 50 μg Con A (Miles-Yeda LTD, Israel) per 30 embryos. Con A was kept in the medium continuously. Simultaneously, control cultures of embryos without Con A in the medium were set up. In order to study the reversibility of the effect of Con A, MAM, at a concentration of 0.05 *M*, was added to cultures of embryos previously treated with Con A for 12 hr. The embryos were kept in MAM for 1 hr, then washed with medium, and cultivated again. The growth of embryos was observed under an inverted microscope and after 24 or 48 hr of incubation, trypan blue exclusion test was carried out to check the embryos' viability.

Results and Discussion. Binding of Con A to unfertilized mouse eggs and embryos. We estimated the mean density of Con A binding sites on unfertilized eggs and on embryos at various stages of development by measuring the radioactivity of ^{125}I-labeled Con A bound to the cells and planimetric evaluation[3] of their surface area. For comparative purposes, the binding sites of normal and transformed somatic cells for Con A were also studied. As shown in Fig. 1, there were no significant differences between vitelluses and embryos at various stages of development in the binding of ^{125}I-Con A. The number of Con A sites available to binding, on vitelluses as well as on embryos, was calculated to be 2.0×10^8. Since there is no significant difference in the surface area of vitelluses and embryos at the 1- or 2-cell stage, we can conclude that the mean distribution of Con A binding sites per unit area is $1.5 \times 10^4/\mu m^2$ for both the vitelluses and embryos. The slight increase in the binding capacity of 6–8-celled embryos (Fig. 1) is probably caused by the increase of the surface area of the embryo. The treatment of embryos with pronase to remove the zona pellucida could, similar to the effect of tryp-

[3] The smallest and largest diameter of the vitelluses, one-celled embryos, and blastomeres of two-celled embryos were measured and the mean value was used to calculate the surface area of the cells.

sin on somatic cells, affect Con A binding (10). In order to exclude this possibility, 2-celled embryos were treated with pronase, incubated for 12 hr, at which time they had developed to the 4-cell stage, and were then exposed to Con A. When tested for ^{125}I-Con A binding capacity, these embryos did not differ from 4-celled embryos treated with pronase immediately before exposure to Con A. The number of binding sites for ^{125}I-Con A available on eggs and embryos was compared to that of somatic cells such as embryo fibroblasts obtained from C57B1/6 mice, normal and transformed by SV40 and C1-1D, derived from L cells. The number of sites available for the binding for somatic cells was calculated to be 3×10^7 for C1-1D, 2×10^7 for C57 normal cells, and 1×10^7 for C57 transformed cells. These values corresponded to those reported by others (11) for somatic cells.

Since the ratio of surface area of vitelluses to that of somatic cells was estimated in our study to be 25–27 to 1, and since it has been suggested that exposure of somatic cells to trypsin increases their Con A binding (12), we concluded that the mean density of Con A sites available to the binding on the surface of the vitellus and the embryo was similar to that of somatic cells.

Agglutination of vitelluses and embryos by Con A. As shown in Table I, preimplantation embryos regardless of their stage of development were agglutinated by Con A at a concentration of 10 μg/ml, whereas vitelluses were agglutinated only after exposure to 2000 μg/ml of Con A. If treatment with pronase was prolonged for 10 min, the vitelluses became agglutinable by Con A at a concentration of 10 μg/ml. In this respect, the behavior of unfertilized eggs after prolonged treatment with pronase may be compared to that of trypsin-treated normal somatic cells which agglutinate after exposure to Con A at concentrations which agglutinate transformed cells (11). After the removal of zona pellucida by mechanical manipulation, it was found that Con A agglutinates the vitelluses again only at a high concentration of 2000 μg/ml.

The effect of Con A on growth of preimplantation embryos. The development of 2-celled embryos is inhibited in the presence of Con A (Fig. 2a), but the effect can be partially reversed by treatment with MAM if the exposure to Con A does not exceed 12 hr (Fig. 2b). Under such conditions, 33% of embryos treated with Con A and subsequently with MAM reached blastocyst stage after 60 hr.

Four-celled embryos treated by Con A underwent one division (Fig. 2C) and were then inhibited in their development, whereas nontreated embryos underwent two divisions in the same time and reached morula stage. The early morulae were also arrested in development by Con A. The untreated morulae (28 of 30) developed into blastocysts within 12–18 hr of cultivation, but those treated with Con A were arrested in pre-

TABLE I. Agglutination of Vitelluses and Embryos by CON A.

	Concentration of Concanavalin A (μg/ml)								
	0	1	5	10	50	100	500	1000	2000
Vitelluses[a]	0	0	0	0	0	0	0	+	++
Vitelluses[b]	0	0	+	++	++	+	++	++	++
Parthenogenetically stimulated vitelluses[c]	0	0	+	++	++	NT[e]	NT	NT	NT
1-celled embryos[d]	0	0	+	++	++	++	++	++	++
2-celled embryos	0	0	+	++	++	++	++	++	++
6–8-celled embryos	0	0	+	++	++	++	++	++	++

[a] The vitelluses from which zona pellucida were removed mechanically behave in the test in the same way.

[b] The vitelluses after the prolonged treatment with pronase.

[c] Parthenogenetically stimulated vitelluses after one or two divisions.

[d] Stage of two pronuclei.

[e] Not tested.

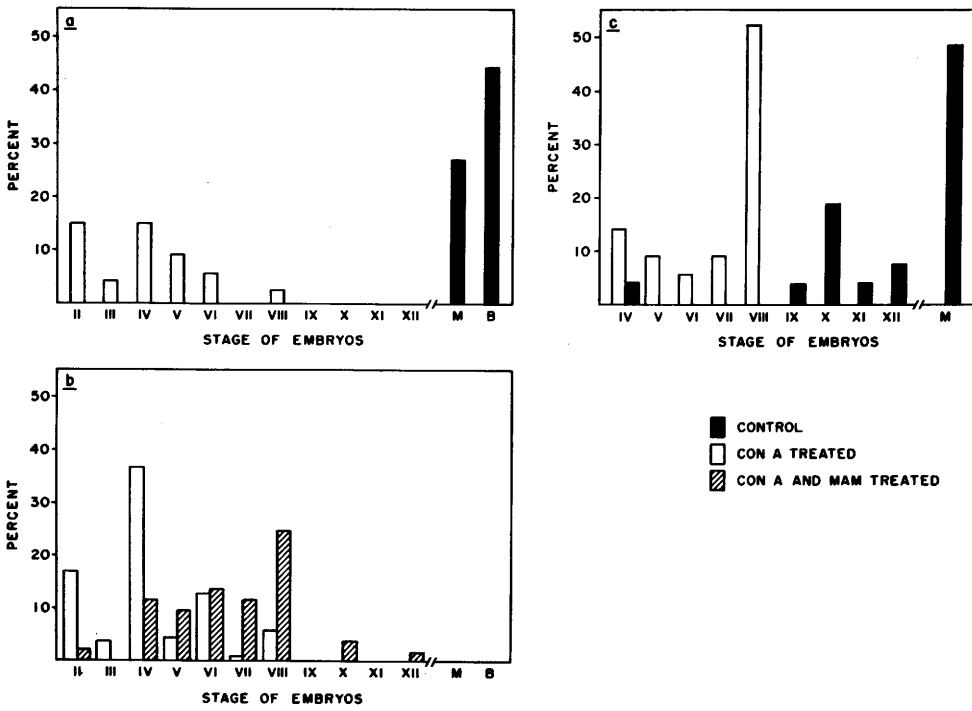

FIG. 2. The effect of Con A on growth of mouse embryos. (a) The percentage of 300 embryos originally obtained, observed at different stages of development after 48 hr in culture with (white bars) or without (black bars) of Con A. Pooled results of five experiments. (b) The percentage of 160 2-celled embryos observed at different stages of development after 12 hr of incubation in the presence of Con A (white bars) and treated afterwards with MAM (dotted bars). Pooled results of two experiments. (c) The percentage of 100 4-celled embryos observed after 24 hr of incubation with (white bars) or without (black bars) Con A. Pooled results of three experiments.

blastocyst stage (only 2 of 30 reached blastocyst stage) when the time of incubation *in vitro* was prolonged up to 30 hr.

The phase of cell cycle of the embryo when Con A was applied seems not to be critical for the effect of Con A to take place. The population of 46–48-hr-old embryos used in our experiments consisted of 2-celled embryos before division and 4-celled embryos right after division.

In contrast to transformed somatic cells for which the native Con A (as used in this study) is toxic under experimental conditions, there was no evidence of toxicity of Con A on embryos. Trypan blue exclusion testing indicated that inhibited embryos were viable 24 and 48 hr after the addition of Con A (Fig. 2).

There is a possibility, however, that the effect of Con A on embryos may be the result, in part, of the agglutination of the embryonic cells by lectin. To rule out this possibility indirectly, we have investigated the effect of Con A bound to Sepharose beads on the development of embryos. Con A–Sepharose B (Farmacia, Uppsala, 4 mg of Con A/ml of swollen beads) was spread on the bottom of culture plates, and 2-celled embryos were layered on top of the beads. Of the 25 embryos adhering to the Con A beads, none divided more than once; 16 of 25 embryos kept on beads without Con A developed to either the blastocyst or morula stage. Similar results were obtained with embryos kept in contact with Con A bound to Sephadex G-50. Since the point of contact between the embryonic cells and either Sepharose or Sephadex beads involves only a small part of the surface of the cell, these results probably indicate that binding

of Con A to a relatively small area of the embryo suffices to cause inhibition of development.

The results presented in this study indicate a marked difference in susceptibility to agglutination by Con A between unfertilized eggs and preimplantation embryos at the earliest stage after fertilization. This difference probably cannot be attributed to the number of sites on cell membranes available to binding by Con A.

The differences in agglutinability between unfertilized eggs (vitelluses) and embryos a few hours after fertilization at the 1-cell stage indicate that a drastic change on the surface of the cell occurs with fertilization. Since there is no difference in Con A binding capacity between unfertilized eggs and embryos, the formation of new binding sites or the unmasking of their cryptic forms upon fertilization seems unlikely. Monroy *et al.* (12) reported a difference in agglutinability between unfertilized and fertilized Ascidian eggs, which is comparable to our result. They suggested that the maturation process, i.e., formation of the second polar body, must be terminated before agglutination occurs. Monroy *et al.* (13) also tested the binding capacity of unfertilized and fertilized Ascidian eggs using fluoresein isothiocyanate-conjugated Con A and found that unfertilized eggs do not bind Con A in contrast to fertilized ones. These results seem to be at odds with our observations, since we have not found a difference in binding capacity between fertilized and unfertilized mouse eggs using ^{125}I-labeled Con A. Use of different markers or a difference between two so-distant species might explain this discrepancy. It is obvious, however, that fertilization, or a process related to fertilization such as parthenogenetic stimulation, caused profound changes in membrane properties which probably allows the formation of agglutination sites. Since similar membrane changes have been observed for transformed somatic cells which lack contact inhibition (10), the agglutinability of preimplantation embryos may indicate that we are dealing here with a parallel phenomenon.

If the agglutination of transformed somatic cells by lectins reflects changes in cell surface properties related to the lack of contact inhibition (5), then we may postulate that following fertilization similar changes take place on the membrane of embryonic cells. The similarity between the growth patterns of preimplantation embryos and transformed somatic cells is evidenced by the fact that Con A inhibits the growth and development of both types of cells.

It would be interesting to know how long the effect of Con A lasts during the process of differentiation of cells of the postimplantation embryos. Retina cells of chick embryos in the later stages of development, when differentiation had already taken place, were found to be as unsusceptible to the effect of Con A as normal adult somatic cells (14).

We had described previously a cytotoxic antibody against unfertilized mouse eggs which will react with cells of preimplantation but not with postimplantation embryos (15, 16). Adult mouse cells are unsusceptible to the cytoxic effect of the serum unless they have been transformed by SV40 (15). The identity of the antigen causing this immunological reaction is still undetermined. Since SV40-transformed cells which are susceptible to agglutination by Con A have a high capacity for growth and show less contact inhibition than normal mouse cells (17), it may be postulated that this antigen may play a role in the contact inhibition phenomenon and that interaction between Con A and the surface of these cells may facilitate study of the antigen.

Summary. Unfertilized eggs and preimplantation mouse embryos had the same binding capacity for Concanavalin A. Concanavalin A in doses of 10 μg/ml agglutinated preimplantation embryos and the embryos derived from parthenogenetically stimulated eggs, but did not agglutinate unfertilized eggs. Unfertilized eggs became agglutinable with Concanavalin A in doses of 10 μg/ml after prolonged pretreatment with pronase or if the concentration of Concanavalin A was raised to 2000 μg/ml. The presence of 50 μg/ml of Concanavalin A in the medium inhibited development of preimplantation mouse embryos. Inhibition

of growth was partially reversible after treatment of Concanavalin A-treated embryos with methyl-α-D-mannopyranose.

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