composed of undifferentiated blast cells, was also able to grow in the absence of insulin, though its growth rate was much slower. The conclusion may be drawn that insulin is not an essential growth factor for most human leukemic cell lines.

The observation that some of these lines are more sensitive to the growth stimulation effects of insulin prompted us to investigate whether this was related to their insulin binding capacity. We and other groups had previously found by competition curve analysis that HL-60 cells possess specific insulin receptors[14-16]. In the present study we demonstrated that all the human leukemic cell lines we tested bind insulin specifically. However, the degree of this activity could not be related to the cell phenotype of the various lines or with their insulin requirement for growth.

It has been shown in other experimental systems that high concentrations of insulin can promote growth in the absence of biologically active insulin[17]. It has also been suggested that insulin's growth promoting activity is distinct from its metabolic activity, and that these activities are mediated by different cell membrane receptors[18].

With the exception of the HL-60 cell line, our study demonstrates that insulin, whether acting as a metabolic agent or as a growth promoter, is not essential for the maintenance of human leukemic cell lines. The presence of specific receptors for insulin on the membranes of cells which are able to grow indefinitely in the absence of the hormone can be regarded as an expression of deranged growth regulatory mechanisms in neoplastic cells.

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Injection of a soluble sperm fraction into sea-urchin eggs triggers the cortical reaction

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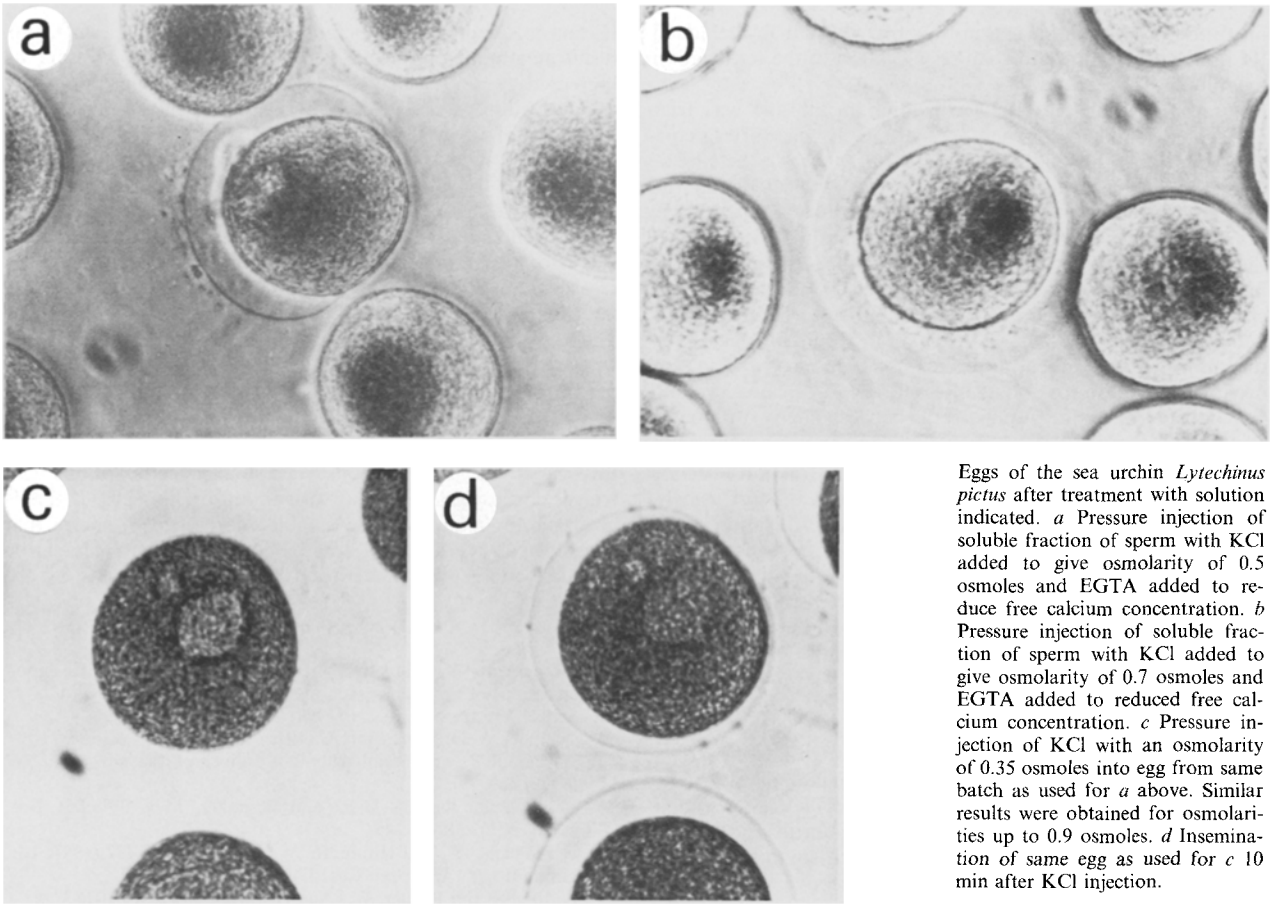
Summary. Fertilization membranes form around unfertilized sea-urchin eggs after microinjection of a soluble spermatozoa fraction isosmotic with seawater. This demonstrates that the spermatozoon contains a chemical that triggers an increase in cytosolic calcium, leading to exocytosis of cortical granules. It also demonstrates that the triggering mechanism does not require an externally-activated egg-membrane process. Further experiments show that the chemical trigger is not calcium.
Key words. Sea-urchin eggs; *Lytechinus pictus;* fertilization membrane; spermatozoa fraction; triggering mechanism.

One of the first events in the normal activation of sea-urchin eggs is exocytosis of the cortical granules leading to formation of the fertilization membrane[1]. Although it has been well established that an increased concentration of cytosolic calcium is necessary for this event[2-5], an important question is how the spermatozoon triggers the calcium increase. We report here that fertilization membranes form around unfertilized sea-urchin eggs after microinjection of a soluble spermatazoa fraction isosmotic with seawater. This indicates that the spermatozoon contains a chemical that triggers an increase in cytosolic calcium, leading to exocytosis of cortical granules. It also demonstrates that the triggering mechanism does not require an externally-activated egg-membrane process.

Injection of live spermatozoa directly into the cytoplasm of sea-urchin eggs does not lead to formation of fertilization membranes[6], indicating that interaction of the spermatozoon with the egg membrane is required to trigger the calcium increase. There are two general types of processes that might be associated with this interaction. One is the entrance of the contents of the spermatozoon into the egg cytoplasm. Another involves egg-membrane-mediated activity, such as the activation that follows binding of a hormone to its receptor. We have addressed the question as to which of these two types of processes are required for cortical granule exocytosis by pressure-injecting a soluble fraction of spermatozoa directly into the cytoplasm of unfertilized eggs and observing whether fertilization membranes form.

All experiments were carried out at room temperature, using gametes of the sea urchin *Lytechinus pictus* (Pacific Biomarine, Venice, California). Egg jelly was removed by washing the eggs several times in natural seawater. To prepare the sperm fractions, the testes of several animals were washed several times in calcium-free seawater, pH 7.8. Dry sperm was collected by pipette, diluted into about three times its volume of deionized water, and homogenized with a teflon rod spinning at 1700 rpm for 2 min. The resulting suspension was centrifuged for 1 h at 110,000 × g at 4 °C and the supernatant collected. Over a period of two months, 9 such fractions were prepared. The osmolarity of these fractions was usually about 0.25 osmoles. The major cations, as determined by atomic absorption spec-



Eggs of the sea urchin *Lytechinus pictus* after treatment with solution indicated. *a* Pressure injection of soluble fraction of sperm with KCl added to give osmolarity of 0.5 osmoles and EGTA added to reduce free calcium concentration. *b* Pressure injection of soluble fraction of sperm with KCl added to give osmolarity of 0.7 osmoles and EGTA added to reduced free calcium concentration. *c* Pressure injection of KCl with an osmolarity of 0.35 osmoles into egg from same batch as used for *a* above. Similar results were obtained for osmolarities up to 0.9 osmoles. *d* Insemination of same egg as used for *c* 10 min after KCl injection.

trophotometry, were Na (80 mM) and K (13 mM). Total calcium concentration ranged from 0.3 mM to 1 mM and the pH ranged from 6.5 to 7.0. In several experiments, EGTA was added to the sperm fraction to chelate the free calcium, using the indicator dye Cal Red[7]. About 10% excess EGTA was added to these fractions, resulting in a concentration of free calcium less than 50 μM: In all experiments with the sperm fraction, before pressure injection, KCl was added to raise the osmolarity to 0.5–0.9 osmoles.

For the pressure injection, conventional patch pipettes of 1–2 μm tip diameter were used. These were back-filled by syringe through a 0.22-μm Millipore filter (Millex-GS). Controlled pressures were provided by a Picospritzer (General Valve Corp., East Hanover, New Jersey). Eggs were placed onto a glass slide to which they adhered loosely. The pipette was pressed against the egg surface with a micromanipulator, and suction was applied until the surface ruptured. This caused a small amount of cytoplasm to enter the tip of the pipette. Then two pressure pulses were applied – the first to reinject the displaced cytoplasm and the 2nd to inject the experimental solution. The injected volume was less than 1% of the egg volume. Assuming a density of 3×10^{10} per ml for dry sperm, this corresponds to less than 15 sperm equivalents per injection. If a fertilization membrane formed, in whole or in part, we recorded the time course of formation and then photographed the egg. Eggs seriously damaged by injection were discarded. In contrast to the behavior of frog eggs, where pricking or other damage often results in raising a fertilization membrane, sea urchin eggs do not readily raise fertilization membranes. In fact, none of these damaged eggs raised fertilization membranes.

For the first six batches of soluble sperm fraction, obtained before the condition of the animals deteriorated late in the season, every one of the 26 eggs injected gave rise to a completely raised membrane or to a raised membrane over a substantial fraction of the egg surface. We observed 17 partial membranes (usually raised about 50%) and 9 complete membranes. A typical partial fertilization membrane is shown in the figure, a, and typical complete fertilization membrane is shown in the figure, b. The time course of fertilization membrane formation is about the same for these injection experiments as for insemination with sperm. After a delay of about 30 sec, the fertilization membrane starts to form near the injection site, and then gradually spreads along the egg surface.

For sperm fractions from animals obtained late in the season, only two partial fertilization membranes developed out of 11 eggs injected, even though control eggs inseminated with sperm from the same batches of animals did raise fertilization membranes. A possible explanation is that these animals contained fewer viable sperm. This would be more critical for sperm-fraction injection experiments, which depend upon obtaining a sufficiently high concentration of active ingredients, than for insemination experiments, where there is normally a large excess of viable sperm.

We also prepared one batch of soluble sperm fraction from *Arbacia* sperm and injected it into *Lytechinus pictus* eggs. The three eggs injected all formed partial fertilization membranes (about 50% of the egg surface).

For each batch of eggs injected with the soluble sperm fraction to which KCl was added to raise the osmolarity, control eggs were injected with pure KCl of the same total osmolarity. In none of the 33 control eggs did the pure KCl solution raise a complete or a partial fertilization membrane (fig., c). After waiting for several min, we then added sperm externally and observed formation of a fertilization membrane around the egg previously injected with KCl (fig., d). These observations dem-

onstrate that the soluble sperm fraction, rather than the added KCl or the act of injection, causes elevation of the fertilization membrane.

Before concluding that the fertilization membrane was triggered by a soluble component of sperm, it is necessary to consider the possibility that the causative agent was injected calcium. Previous injection experiments have indicated that the threshold for obtaining elevation of the fertilization membrane is very dependent on whether or not calcium buffer is added to the injectate[5]. Presumably, when the calcium is unbuffered, the internal calcium concentration is reduced by the natural calcium sequestering system of the cells. When calcium buffer is added, the threshold is 0.2 μM calcium in sea-urchin eggs[5]. The threshold without added buffer has not been measured in sea-urchin eggs, but in frog oocytes it is 0.3 μg of calcium[8]. Taking into consideration the volume ratio between the two preparations, that corresponds to about 300 pg for sea-urchin eggs. The amount of calcium in our soluble sperm fraction injectate was less than 0.1% of this amount (less than 0.2 pg), and is, therefore, unlikely to cause elevation of the fertilization membrane. We tested this point directly, however, by injecting into sea-urchin eggs between 0.2 and 2 pg of unbuffered calcium to which KCl was added to adjust the osmolarity. In this range of calcium, in 17 trials there was no response, and in three trials there was only an unpropagated local response over less than 10% of the egg surface. This demonstrates that the calcium in our soluble sperm fraction is not the trigger for propagation of cortical granule exocytosis.

This conclusion is consistent with two other results. When we reduced the free calcium concentration in the soluble sperm fraction to less than 50 μM by adding EGTA, this soluble sperm fraction was as effective in raising fertilization membranes as was the fraction without EGTA. Also, in the batches of soluble sperm fraction that were ineffective, the calcium concentration was the same as in those that were effective.

Since the soluble sperm fraction caused partial or full elevation of the fertilization membrane in every egg of the first six batches into which it was injected, and control injections of KCl never caused any elevation of the fertilization membrane, we conclude that the soluble sperm fraction contains a trigger for the initiation of cortical granule exocytosis. Although these results do not completely rule out the possibility that the trigger reacts with the egg membrane, they do provide evidence that any putative egg-membrane-mediated reaction is not activated from the external side of the membrane. This conclusion is based on our experiments demonstrating activity when the sperm factor is injected directly into the cytoplasm and on Hiramoto's experiments[6] demonstrating that the sperm factor is not membrane-permeable.

Increased cytosolic calcium is responsible for other important egg-activation processes in addition to cortical granule exocytosis. One of these is an increase in intracellular pH, leading to cytoskeletal reorganization, increased protein synthesis, and initiation of DNA synthesis[9]. Another is the activation of NAD kinase, leading to the regulation of sugar metabolism[9]. Thus, fertilization-membrane formation can be regarded as an assay for the more general processes of cytosolic calcium increase and egg activation. A likely source of internal calcium is the tubular reticulum recently described[10].

The idea that cortical granule exocytosis and egg activation are triggered by a component of sperm that enters the egg cytoplasm was suggested more than 70 years ago[11,12]. In other models for triggering, the effect of the spermatozoon on the external surface of the egg membrane was emphasized[13,14]. In this paper we have demonstrated that injection alone can trigger cortical granule exocytosis in sea-urchin eggs. If this is also true for mammals, the soluble component of sperm that triggers exocytosis, leading to a block to sperm entry, might be useful as an agent to prevent fertilization.

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Nucleolus DNA synthesis in *Vicia faba* root-tip meristems

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Summary. Autoradiographs of *Vicia faba* lateral root-tip meristems were prepared following a 30 min pulse with ^{3}H-thymidine. 1/3 of all interphase nuclei in the meristem are labeled, most with a uniform distribution of silver grains. 2–5% of labeled nuclei show specific nucleolus DNA labeling.

Key words. Vicia faba; nucleolus; amplification; ^{3}H-thymidine; ribosomal DNA.

The existence of developmentally regulated gene amplification is well known. The nucleolar rDNA of the *Xenopus* oocyte nucleus is some 1400–2000-fold more abundant than the rDNA of a somatic nucleus[1]. DNA puffs have been studied extensively in *Rhynchosciara*[2-4]. More recent studies of insect chorion multigene families have revealed similar results: in *Drosophila* only 1–3 copies of one gene set are present in germ and somatic cells, but they are amplified up to 25 copies in the ovarian follicle cells producing chorion proteins[5]. Amplification of actin gene sequences has been discovered during chick myogenesis[6]. In addition, recent studies of cultured animal and human cells have related resistance of the antitumor drug methotrexate to spontaneous gene amplification[7].

The present study reports the presence of specific nucleolus