EFFECT OF PROTEOLYTIC ENZYMES ON THE ULTRASTRUCTURE OF ANTIBODY-TREATED SEA URCHIN EGGS

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Immunofertilization studies have shown that antisera have a number of effects on sea urchin eggs. These include morphological alterations (Perlmann, 1954; Tyler and Brookbank, 1956) and inhibition of the fertilizability of the treated eggs (Perlmann, 1954, 1959). An unusually interesting feature of the action of antibody on the fertilizing capacity of the eggs is the dependence on the bivalent character of the antibody. Treatment of the eggs with univalent antibody prepared by papain digestion has no effect on fertilizability (Metz and Thompson, 1967; Graziano and Metz, 1967). The failure of univalent antibody to inhibit the fertilizability of eggs is interpreted to mean that there are no essential fertilization antigens at or near the egg surface, at least in the sense of antigenic combining sites that must be free to function in initial stages of fertilization. Inhibition of fertilization by bivalent antibody must then depend upon secondary or tertiary effects associated with the capacity of bivalent antibody to cross-link neighboring antigens. It then becomes of interest to determine what such secondary factors might be. One possibility (Baxandall, 1966) is that the 0.1 μ thick surface layer of electron-opaque material reported by Baxandall et al. (1964) in electron micrographs of bivalent antibody-treated eggs is a barrier to sperm-egg interaction. This view is consistent with the fact that the layer is not found on univalent antibody-treated eggs (Metz et al., 1967; Metz, 1968). Finally, bivalent antibody-treated eggs recover fertilizability when posttreated with proteolytic enzymes (Metz and Thompson, 1967; Graziano and Metz, 1967). In the present study such eggs were examined with the electron microscope in a further attempt to correlate the electron-opaque layer with fertilization inhibition by bivalent antibody.

MATERIAL AND METHODS

The sea urchins, *Lytechinus variegatus*, were collected in Biscayne Bay, Dade County, Florida. Gametes were obtained by KCl-induced shedding. Antisera were prepared in three different rabbits by injecting them with homogenate of acid dejellied eggs in Freund's adjuvant as previously described (Metz and Thompson, 1967). The γ-globulin fraction was

TABLE I

Response of Antibody-Pretreated, Protease-Digested Lytechinus Variegatus Eggs to Insemination

	Anti-jellyless *Lytechinus* egg rabbit γ-globulin-pretreated eggs.		Control rabbit γ-globulin-pretreated eggs.	
	Protease	Seawater	Protease	Seawater
% Cleavage	64	0	77	70
% Cleaved plus uncleaved polyspermic eggs	100	0	92	80

Eggs were exposed to anti-jellyless egg homogenate rabbit γ-globulin (rabbit No. 6511) or control rabbit γ-globulin (rabbit No. 6511) for 30 min, were washed, and treated with 0.1% protease or seawater for 15 min and again washed in seawater.

For insemination 2 drops of concentrated eggs were transferred to 5 ml of *Lytechinus* semen previously diluted to 0.33% with seawater. 90 min after insemination, the eggs were examined with light optics and scored (>100 eggs counted) for cleavage, multiple asters (pathological polyspermy), and eggs with intact nucleus and no asters. The latter eggs were regarded as unfertilized.

partially purified by precipitation at 18% Na_2SO_4. The material was then dialyzed against seawater.

In the experiments, 1 volume (32 drops) of acid dejellied eggs was mixed with 2 volumes (64 drops) of control and of immune γ-globulin. After 30 min the eggs were washed twice in 10-ml volumes of sea-water and concentrated. One-half of the concentrated immune and control γ-globulin-treated eggs were transferred to 5 ml of 0.1% protease (a pancreatic extract prepared by Nutritional Biochemicals Corporation, Cleveland, Ohio) solution in seawater. The remaining half of the immune and control γ-globulin-treated eggs were placed in 5-ml samples of seawater. After 15 min all the eggs were washed three times in 10-ml volumes of seawater. Aliquots of each egg sample were then inseminated for subsequent evaluation of fertilizability and fixed for ultrastructural examination at approximately the same time. Initial fixation was done for 2 hr in paraformaldehyde-glutaraldehyde (Karnovsky, 1965). After they had been washed with 0.1 M phosphate buffer (pH 7.5), the eggs were post-fixed in phosphate-buffered 1% osmium tetroxide, dehydrated through graded concentrations of ethanol, and subsequently embedded in Araldite (Durcupan; Fluka AG, Basel, Switzerland). Thin sections, stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965), were examined in a Philips 300 electron microscope.

RESULTS

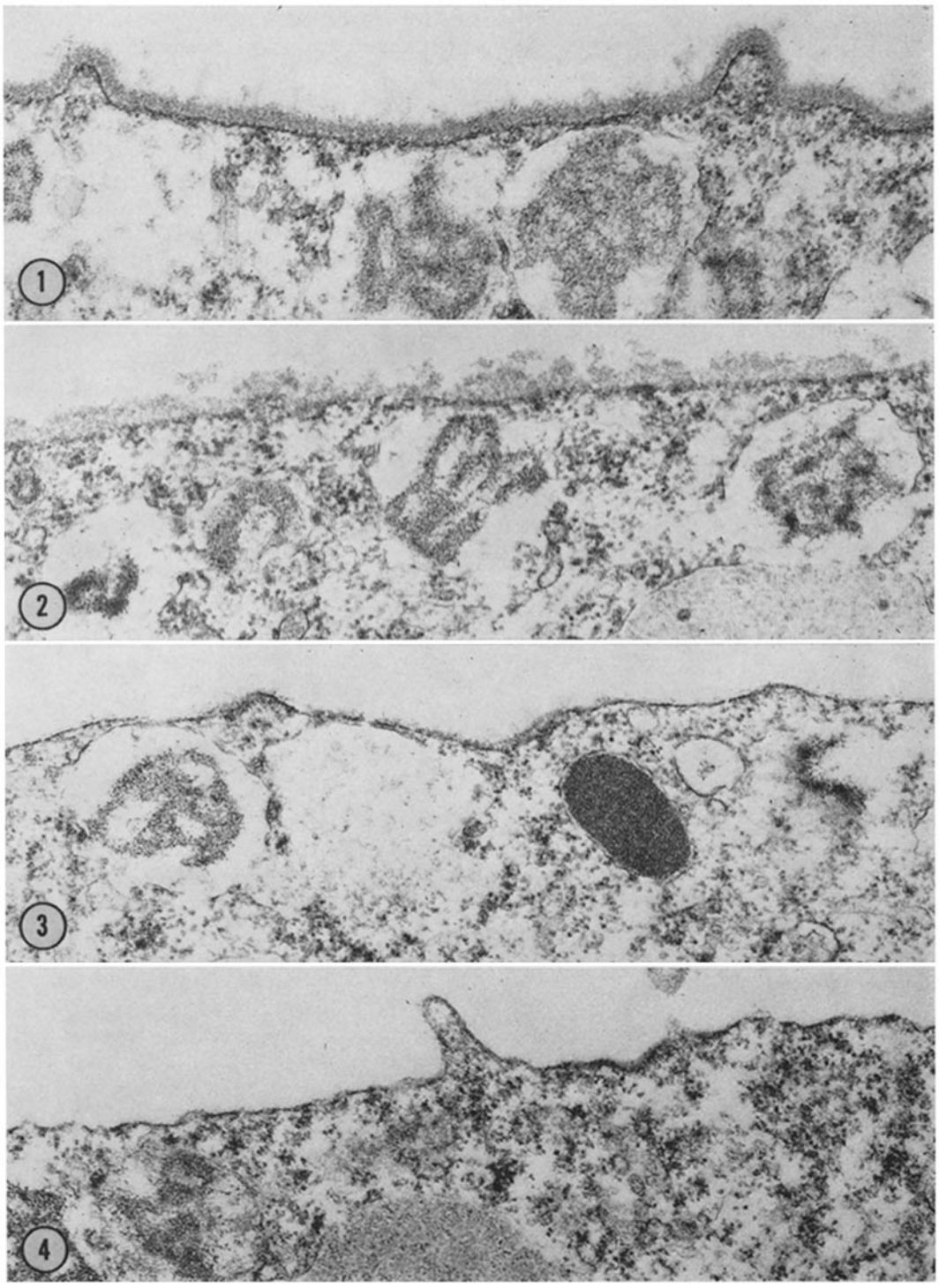
Three experiments yielded a high percentage of fertilization (> 60% cleavage) of the antibody–protease-treated eggs and sufficient egg recovery for fixation. The fertilization data and electron micrographs from one of these experiments are given in Table I and Fig. 1–4, respectively.

The values for per cent cleavage (Table I) reflect minimal values for per cent fertilization. This is especially true for the protease-treated samples because of the high incidence of pathological polyspermy and associated cleavage inhibition known to result in proteolytic enzyme-treated eggs (Tyler et al., 1956). Therefore, values for cleaved plus uncleaved polyspermic eggs, e.g. total per cent fertilization, are also given in Table I. Clearly the protease treatment restored fertilizability to the antibody-pretreated eggs in this experiment.

The electron micrographs show the thick (e.g. 0.1 μ) electron-opaque layer at the surface of the antibody-treated, seawater-posttreated eggs (Fig. 1) as described by Baxandall et al. (1964) and by Metz et al. (1967, and see Metz, 1968). This layer is absent from all control γ-globulin-treated eggs (Figs. 3 and 4).

Finally, the antibody-treated, protease-posttreated eggs do possess some surface material not evident on the control γ-globulin-treated eggs (Fig. 2). However, this layer is less dense and less adherent to the egg surface than the layer on antibody-treated, seawater-posttreated eggs. In addition the layer on the protease-posttreated eggs is not uniform in width and contains breaks or discontinuities.

Comparison of micrographs from the three experiments shows some variability in the degree of alteration of the dense surface layer in the antibody–protease-treated eggs. This is not surprising since different samples of antisera were used and the length of exposure to protease varied slightly. In any event, the extremes of variability in the three experiments were approximately the same as the variability among different antibody-protease–treated eggs within the individual experiments. In all cases the antibody-protease–treated eggs were readily distinguishable from the antibody-seawater–treated eggs.



FIGURES 1–4 *Lytechinus variegatus* eggs from aliquots fixed immediately prior to insemination of experimental samples (Table I). All figures: × 21,000. Fig. 1 shows antibody-treated eggs, posttreated with seawater. An electron-opaque layer of uniform width is adherant to the egg surface. Fig. 2 shows an antibody-treated egg, posttreated with protease. The surface layer is less electron opaque than that of Fig. 1, exhibits variations in width, and discontinuities. Fig. 3 is of a control γ-globulin-treated egg, posttreated with seawater. The surface layer is absent. Fig. 4 shows a control γ-globulin-treated egg, posttreated with protease. The surface layer is absent.

DISCUSSION

This study shows quite clearly that the electron-opaque layer at the surface of bivalent antibody-treated eggs is subject to attack by protease. The effects of the protease, as visualized with the electron microscope, correlate well with recovery of fertilizability of the eggs. This correlation is consistent with the view that the electron-opaque egg surface layer produced by bivalent antibody treatment inhibits fertilization by mechanically blocking sperm-egg interaction as is suggested by Baxandall (1966). The partial removal of the dense layer by the protease treatment would seem to be sufficient to account for restoration of fertilizability in this case. However, this interpretation may not be generally applicable because, in *Arbacia*, restoration of fertilizability to antibody-pretreated eggs by protease depends upon the particular anti-egg antibody employed (Graziano and Metz, 1967).

Finally, it should be noted that proteolytic enzyme treatment removes the vitelline membrane from sea urchin eggs (see Tyler and Metz, 1955). A block to fertilizability could reside at this level. Since bivalent antibody inhibits fertilizability of both control and protease-pretreated (vitelline membraneless) eggs whereas univalent antibody does not (Metz and Thompson, 1967), blocks to fertilization in either the presence or absence of the vitelline membrane must necessarily result from steric masking of nonantigenic sites by bivalent antibody. Such sites could be masked by the electron-opaque layer that results from bivalent antibody treatment and "unmasked" by the protease treatment as described in this study.

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