Studies on the binding of antibody against mouse lactate dehydrogenase (isoenzyme X) by preimplantation mouse embryos

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Summary. The binding of antibodies against LDH-X by preimplantation mouse embryos was studied to detect LDH-X from spermatozoa in embryos after fertilization. Incubation of preimplantation mouse embryos with rabbit anti-mouse-LDH-X-IgG and then with peroxidase-labelled goat anti-rabbit IgG revealed a strong peroxidase staining of the zona pellucida of normal fertilized and unfertilized 1-cell ova. However, the reaction was significantly weaker with both fertilized and unfertilized 1-cell ova from females induced to superovulate and normal and superovulated blastocysts. Pure antibody against mouse LDH-X was obtained by affinity chromatography of the rabbit anti-mouse LDH-X-IgG on pure mouse LDH-X covalently bound to sepharose. The pure antibody against mouse LDH-X reacted immunochemically identically to anti-mouse LDH-X-IgG, but it was not bound by any stage of preimplantation mouse embryos. The IgG fractions which had passed through the affinity column during the purification procedure and which did not contain any anti-LDH-X activity were bound by the zonae of preimplantation mouse embryos in the same manner as was unpurified anti-mouse LDH-X-IgG. Histochemical studies indicated LDH activity only in the embryo proper, but not on the zona pellucida. It is concluded that LDH-X is not present in preimplantation mouse embryos.

Introduction

An inhibition of pregnancy in mice and rabbits by active and passive immunization with the X isoenzyme of mouse lactate dehydrogenase (LDH-X) has been reported by Goldberg & Lerum (1972), Goldberg (1973) and Lerum & Goldberg (1975). These investigators interpret their results in terms of a reaction of anti-LDH-X with LDH-X released from degenerated supernumerary spermatozoa in the perivitelline space, resulting in delayed mortality. Evidence for a localization of LDH-X within the zona pellucida and on the surface of the embryo proper has been presented by the same group with peroxidase-conjugated goat anti-rabbit IgG after incubation of mouse blastocysts with rabbit anti-LDH-X-IgG (Bene & Goldberg 1974). Unfertilized ova after superovulation, however, did not bind antibody to LDH-X. In additional studies, using fluorescein-conjugated goat anti-rabbit IgG, the intense fluorescence of the zona pellucida but not of the embryo confirmed the contention that blastocysts can bind antibody to LDH-X (Goldberg, 1974; Wheat & Goldberg, 1975).

Because we have not been able to detect any LDH-X in preimplantation mouse embryos, and as our experiments indicated an effect of the antibody to LDH-X on mouse gametes, but not on early development (Erickson, Hoppe, Tennenbaum, Spielman & Epstein, 1975a; Erickson, Spielmann, Mangia, Tennenbaum & Epstein, 1975b), we tried to repeat the studies demonstrating the presence of receptors for the antibody to LDH-X on or within the preimplantation mouse embryo after fertilization. Moreover, because we were not convinced that embryos derived from eggs released normally and after superovulation have identical surface components, we studied the binding of the antibody to LDH-X by all developmental stages of preimplantation mouse embryos. To support our data on the localization of LDH-X, the direct histochemical staining method for the detection of LDH activity was used on preimplantation mouse embryos.

Materials and Methods

Mice of the NMRI strain (Schwenke & Co., Nauheim, West Germany) were used in all experiments. Immature females were induced to superovulate by intraperitoneal injections of 5 i.u. PMSG followed 48 h later by 5 i.u. HCG (Schering A.G., West Germany). To obtain cleavage-stage embryos untreated and hormone treated females were placed with males overnight. The presence of a copulatory plug the next morning indicated Day 0 of pregnancy. At appropriate times the various developmental stages were obtained by flushing oviducts or uteri with Whitten's Medium for Ovum Culture (WMOC: Whitten, 1971). Embryos were obtained at the following times after the midpoint of the dark period: 1-cell stages, 12 h; 2-cell stages, 36 h; 8-cell stages, 60 h; morulae, 84 h; blastocysts, 96 h. A 10-min incubation at 37°C with 150 units hyaluronidase (Boehringer, Mannheim, West Germany) removed the cumulus cells from the 1-cell ova and from unfertilized ova of hormone-treated females which had not been mated. Unfertilized normal ova were obtained by dissecting ovaries in WMOC without any hyaluronidase. In a few experiments on zona-free 1-cell ova, 300 units pronase (Boehringer, Mannheim, West Germany) in WMOC were used to disperse the cumulus cells and to dissolve the zona.

LDH-X was purified from mouse testes by two steps of affinity chromatography on oxamate-sepharose. At concentrations of 200 μ M-NADH and 0.5 M-NaCl LDH-X is first separated from the other LDH isoenzymes of mouse testis on oxamate-sepharose (Spielmann, Erickson & Epstein, 1973). In the second step, LDH-X is bound to the same matrix at lower NADH and NaCl concentrations and the pure enzyme (specific activity: 120 i.u./mg) can be subsequently eluted in the absence of NADH as outlined elsewhere (Spielmann, Eibs & Mentzel, 1976).

For the production of an antibody against mouse LDH-X polyacrylamide was used as adjuvant to increase the antigenic activity of the enzyme (Spielmann, Erickson & Epstein, 1974). Acrylamide slices containing an estimated 75 μ g LDH-X protein were pulverized in saline and homogenized with equal volumes of complete Freund's adjuvant for the first subcutaneous injection and with incomplete Freund's adjuvant for subsequent s.c. injections. The animals were bled at weekly intervals after the fourth weekly injection, and booster injections were given monthly. The antiserum was pooled from the 4th–6th bleedings. It showed a single precipitin line when reacted with testicular extracts, and this band of precipitation fused with the band obtained when the antiserum was tested with pure LDH-X. There was no cross-reaction with crude extracts of other organs, and absorption with such extracts did not remove the precipitation arc. Our antiserum to LDH-X had a lower inactivation titre (Erickson *et al.*, 1975a) than that reported by Goldberg & Lerum (1972): an average 100% inactivation titre of 0.15 ml/unit compared to 0.0059 ml/unit. Gamma globulin fractions were obtained from rabbit anti-LDH-X serum and from control serum by ammonium sulphate precipitation (33% saturation), taken up in 0.05 M-sodium phosphate buffer at pH 7.5, dialysed against the same buffer, and after adjustment of the protein concentration to 20 mg/ml stored at -20°C.

Further purification of the anti-LDH-X-IgG fraction was attained by immunoabsorption on a column of pure mouse LDH-X covalently bound to sepharose 4B (Sigma Chemical Co., Munich,

EXPLANATION OF PLATE 1

Fig. 1. Immunohistochemical staining of preimplantation mouse embryos for peroxidase reaction product after preincubation with anti-LDH-X-IgG and then with anti-IgG peroxidase. The 6 normal 1-cell ova (3 fertilized and 3 unfertilized) show a positive reaction (dark staining) indicating receptor for anti-LDH-X-IgG; the 6 1-cell ova from a superovulating female (3 fertilized and 3 unfertilized) and 3 blastocysts (large embryos) all have a negative reaction (weak staining). $\times 130$.

Fig. 2. Incubation procedure identical to that described in Fig. 1; the 2 normal 1-cell ova show a positive reaction; 3 empty zonae pellucidae obtained from normal 1-cell ova by mechanical treatment show a positive reaction; 3 nude blastocysts after mechanical hatching are negative. $\times 130$.

Fig. 3. Normal 1-cell ova and 2-cell embryos were incubated to demonstrate LDH by the presence (dark staining) of formazan granules. Without previous fixation: negative reaction in zona pellucida and embryonic cells (3 1-cell ova and 3 2-cell embryos). After 20 min fixation in formalin: reaction negative in zona pellucida but positive in the embryonic cells (3 1-cell ova and 3 2-cell embryos). $\times 130$.

Fig. 4. Ouchterlony double-diffusion test. Well 1, mouse testis homogenate; Well 2, saline control; Well 3, pure anti-LDH-X; Well 4, unpurified anti-LDH-X-IgG; Well 5, anti-LDH-X-IgG fraction which passed through LDH-X-sepharose column without being bound.

West Germany). The sepharose was activated by the cyanogen bromide procedure (Cuatrecasas, 1970). Highest yields of matrix-bound enzyme activity were obtained by reducing the amount of cyanogen bromide (Serva Chemie, A.G., Heidelberg, West Germany) to 15 mg/ml sepharose (Levi, 1975). Pure LDH-X (approximately 3 mg/ml) was dissolved in 0.01 M-sodium bicarbonate, pH 8.0 (Livingston, 1974), activated sepharose was added, and the sepharose-protein suspension was mixed at 4°C for 18 h. The LDH-X/spharose ratio was approximately 10 mg/ml. When a solution of 0.3 mM-NADH and 4 mM-pyruvate in 0.01 M-tris-HCl, pH 8.0, was passed through the column, rapid and complete oxidation of the coenzyme occurred as indicated by a decrease in optical density at 340 nm (Hofstee, 1973). Thus the enzyme was firmly bound to the sepharose and the active site was available to the substrate.

The purification of the antibody on the matrix-bound LDH-X followed the procedure used by Olsen & Prockop (1974). The sepharose-bound LDH-X was poured into a column that was equilibrated at 4°C with 0.1 M-sodium phosphate buffer, pH 7.3. Then 3 ml rabbit anti-LDH-X-IgG (20 mg/ml) were passed through the column and the column was washed with several volumes of 0.1 M-sodium phosphate buffer. Pure anti-mouse LDH-X was eluted with 3 M-KSCN and desalted by dialysis at 4°C against several changes of 0.1 M-sodium phosphate buffer, pH 7.3. The IgG fraction, which passed unbound through the column, did not contain any antibody against mouse LDH-X, as judged from the Ouchterlony double-diffusion test (Pl. 1, Fig. 4) and from inactivation studies which were performed as previously described (Spielmann *et al.*, 1974). The pure anti-mouse LDH-X, however, contained the total antibody activity against LDH-X as indicated by the same criteria (see Pl. 1, Fig. 4).

Absorption of the anti-LDH-X-IgG fraction was performed with lymphocytes which had been prepared from spleen cell suspensions as described by Ford & Hunt (1973). The incubation procedure for the detection of anti-LDH-X-IgG binding sites on the surface of the embryos was the same as described by Bene & Goldberg (1974). We also used 4-chloro-1-naphthol-H₂O₂ (Serva Chemie A.G., Heidelberg, West Germany) as substrate (Nakane, 1968) for horseradish peroxidase conjugated to goat anti-rabbit IgG (Miles Laboratories, Slough, England).

Histochemical localization of LDH in the various developmental stages of the embryos was performed as described by Solter, Damjanov & Skreb (1972), after a 20-min formalin fixation and without any pretreatment, in incubation medium (Wachsmuth, Zimmermann & Schmidt, 1969) with both lactate and valerate as substrates.

Empty zonae were prepared by suction of embryos into pipettes with a diameter smaller than that of the embryos. The zonae were ruptured by this treatment and the embryos of the early cleavage stages were destroyed. Nude morulae and blastulae, however, with their smaller blastomeres, remained intact. Ruptured zonae of all developmental stages and nude morulae and blastulae were used in the incubation experiments.

EXPLANATION OF PLATE 2

Fig. 5. Peroxidase reaction of normal fertilized 1-cell ova after incubation with anti-IgG-peroxidase. The 3 ova preincubated with anti-LDH-X-IgG showed a positive reaction (see Fig. 1) and the 3 ova preincubated with anti-LDH-X-IgG fraction which was not bound by LDH-X-sepharose also showed a positive reaction. The 3 ova preincubated with control serum IgG and the 3 ova preincubated with pure anti-LDH-X gave negative reactions. $\times 130$.

Fig. 6. Peroxidase reaction of preimplantation mouse embryos after incubation with pure anti-LDH-X and then with anti-IgG-peroxidase. The 5 normal fertilized 1-cell ova and the 5 normal blastocysts all gave negative reactions. $\times 130$.

Fig. 7. Incubation procedure as for Fig. 6 except that the first preincubation step was performed with the anti-LDH-X-IgG fraction which passed through the LDH-X-sepharose column without being bound. Top, 4 normal 1-cell ova demonstrating a positive reaction; lower right, 4 fertilized 1-cell ova from a super-ovulating female showing a negative reaction; centre, 4 normal blastocysts showing a negative reaction.

Fig. 8. The peroxidase reaction of normal fertilized 1-cell ova after incubation with anti-IgG-peroxidase. The 5 ova preincubated with anti-LDH-X-IgG gave a positive reaction; the 5 ova preincubated with anti-LDH-X-IgG after absorption with mouse lymphocytes showed a negative reaction. $\times 130$.

Results

Normal ovarian oocytes and fertilized 1-cell ova bound the anti-LDH-X-IgG considerably more strongly than did fertilized and unfertilized 1-cell ova from superovulating females (Pl. 1, Fig. 1). Fertilization did not affect the binding in either of the two groups. Normal blastocysts showed a significantly weaker staining than normal 1-cell ova, although in normal cleavage-stage embryos the binding of anti-LDH-X-IgG remained constant or decreased only slightly between the 1-cell and the morula stage, as indicated by peroxidase reaction product. The weak binding of anti-LDH-X-IgG to the cleavage stages of embryos from hormone-treated females did not change during the pre-implantation period.

Mechanically isolated empty zonae pellucidae of 1-cell ova and 2-cell embryos reacted as strongly with anti-LDH-X-IgG as did intact embryos (Pl. 1, Fig. 2) but anti-LDH-X-IgG was weakly bound by the zonae pellucidae and not at all by the surface of nude blastocysts which had hatched normally or after mechanical rupture (Pl. 1, Fig. 2). After incubation of preimplantation mouse embryos with pronase to dissolve the zona, anti-LDH-X-IgG was no longer bound by the zona and after subsequent lysis of the zona no binding occurred to the surface of the embryo. The pronase treatment apparently destroys the receptor for anti-LDH-X-IgG on the surface of the zona. We were unable to detect any peroxidase reaction products in the perivitelline space of preimplantation mouse embryos.

Since many groups have previously failed to detect electrophoretically any LDH-X in extracts of preimplantation mouse embryos (Erickson *et al.*, 1975b), we examined whole embryos histochemically for LDH activity with both lactate and valerate as substrates, the latter being characteristic of LDH-X (Allen, 1961). In contrast to the strong binding of anti-LDH-X-IgG to the surface of the zona pellucida, formazan granules, as reaction products of LDH activity in histochemical studies, were never observed in or on the zona, but only in the embryonic cells, where they were evenly distributed (Pl. 1, Fig. 3). When valerate was used as substrate in the incubation mixture, no staining occurred in the embryonic cells and there was no reaction product visible on the zona.

We had some doubts about the specificity of the strong binding of anti-LDH-X-IgG to normal 1-cell mouse ova, because this reaction was not inhibited by absorption with pure mouse LDH-X. In addition, the localization of a receptor for the anti-LDH-X-IgG on the zona pellucida of these embryos is in contrast to the histochemical findings (Pl. 1, Fig. 3). We therefore attempted a further purification of anti-LDH-X-IgG by affinity chromatography on the matrix-bound antigen as described in the 'Materials and Methods'. The IgG fractions which were not bound by the affinity column did not contain any anti-LDH-X activity (see Pl. 1, Fig. 4) and served as controls in the studies on the binding of pure anti-LDH-X by preimplantation mouse embryos.

Pure anti-LDH-X serum did not react more strongly with normal 1-cell ova than did control serum IgG (Pl. 2, Fig. 5). However, the fraction of anti-LDH-X-IgG which was not bound by the sepharose column reacted as strongly with normal 1-cell ova as did unpurified anti-LDH-X-IgG (Pl. 2, Fig. 5). In this part of the investigation the anti-LDH-X titre, determined by immunotitration studies (method described by Spielmann *et al.*, 1974), was identical for anti-LDH-X-IgG and pure anti-LDH-X, but no anti-LDH-X activity could be detected in the anti-LDH-X-IgG fraction which passed through the LDH-X-sepharose without being bound. Additional incubation studies demonstrated that pure anti-LDH-X-IgG is not specifically bound by any developmental stage of normal preimplantation mouse embryos, i.e. the binding to normal 1-cell ova and normal blastocysts is negative (Pl. 2, Fig. 6). The fraction of anti-LDH-X-IgG which was not bound by the LDH-X sepharose and which did not contain any antibody against LDH-X was bound by preimplantation mouse embryos in the same manner as was unpurified anti-LDH-X-IgG (Pl. 2, Fig. 7).

We have previously been unable to detect more than one band in the Ouchterlony double-diffusion test with anti-LDH-X-IgG and no band when testing the fraction which was not bound by the LDH-X-sepharose (Pl. 1, Fig. 4), although this fraction reacted very strongly with normal 1-cell ova (Pl. 2, Figs 5 and 7). We therefore assumed that this reaction was caused by a non-precipitating antibody in the anti-LDH-X-IgG fraction. To test this assumption, anti-LDH-X-IgG was absorbed with mouse lymphocytes. The absorbed anti-LDH-X-IgG fraction reacted in the double-diffusion test and also in the incubation studies with preimplantation mouse embryos in the same way as did

pure anti-LDH-X: it still contained the antibody against LDH-X, as indicated by the double-diffusion test, but did not contain the soluble contaminating antibody with the strong affinity to the zona of normal 1-cell ova (Pl. 2, Fig. 8).

Discussion

In our first attempts to confirm the data of Bene & Goldberg (1974), we found, in agreement with these authors, receptors for anti-LDH-X-IgG in some stages of preimplantation mouse embryos, but in contrast to their results we detected the binding sites on the surface of the zona pellucida of 1-cell ova even before fertilization. In two studies of the binding of fluorescein-labelled goat anti-rabbit IgG (Goldberg, 1974; Wheat & Goldberg, 1975), the binding was described as being considerably stronger to the zona than to the blastocysts and we have also found this in the present study. This weak binding to the embryo proper has not been sufficiently explained. The apparent difference in the number of binding sites for anti-LDH-X-IgG in 1-cell ova from normally ovulating and super-ovulating females in our experiments has not been described by previous investigators. As there is no difference in the binding pattern of anti-LDH-X-IgG to fertilized and unfertilized ova of whatever background, it seems very unlikely that binding sites for anti-LDH-X-IgG are introduced by spermatozoa after fertilization.

Our studies with pure anti-LDH-X provide no evidence for the presence of LDH-X-like receptors on the surface of either the zona pellucida or on the embryo proper. This is in complete accord with our histochemical studies (Pl. 1, Fig. 3) which, in contrast to the findings of Bene & Goldberg (1974), do not indicate any LDH activity either in or on the surface of the zona pellucida or in the perivitelline space.

The strong affinity of anti-LDH-X-IgG to the zona pellucida of normal 1-cell ova could not be removed by absorption with pure LDH-X and it was also present in that part of the anti-LDH-X-IgG fraction which did not contain antibody activity against LDH-X after the purification step. This unexpected binding pattern may indicate that a second, non-precipitating (natural) antibody was elevated in titre by the immunizations and could only be detected by the indirect immunohistochemical studies with anti-IgG-peroxidase. In our previous experiments (Erickson *et al.*, 1975a, b) there was never any indication for the presence of antibodies other than anti-LDH-X in the unpurified anti-LDH-X-IgG, findings corroborated by the double-diffusion test (Pl. 1, Fig. 4) in the present study which demonstrates that after affinity chromatography of anti-LDH-X-IgG on LDH-X-sepharose there is apparently no antibody detectable in the unbound IgG fractions. The same fractions, however, seem to contain the non-precipitating antibody, as demonstrated by the strong binding of these fractions to 1-cell ova in the incubation studies with anti-IgG-peroxidase. A similar non-precipitating antibody may also have caused the strong binding of anti-LDH-X-IgG to blastulae in the study of Bene & Goldberg (1974). From the data gathered from our studies we should like to propose that the IgG fractions containing antibody activity should be purified as thoroughly as possible before being used in indirect immunohistochemical studies with anti-IgG-peroxidase.

We have been using an anti-LDH-X-IgG throughout our studies that had a considerably lower inactivation titre than that used in similar experiments by Goldberg's group. We have overcome this disadvantage by performing additional control incubation experiments with various developmental stages of preimplantation mouse embryos and particularly by purifying the antibody on the antigen affinity chromatographically.

The peroxidase staining method is rather time consuming when using preimplantation embryos, as a very tight schedule of washings and incubations has to be followed. It was, therefore, impossible to carry out more than 6 different single experiments during one series of incubations. For an evaluation of the results of an incubation experiment, we took photographs of randomly picked embryos of each group. Since the staining media in our study were prepared fresh every day, there was always some variation in the staining intensities of peroxidase reaction product and therefore positive and negative embryos had to be expected between different experiments. Furthermore, we did not attempt to compare positive and negative staining results of different developmental stages in detail, since the basic result of this report is the lack of LDH-X-like binding sites on preimplantation mouse embryos when the experiments are carried out carefully.

We have reported a significant decrease in litter size after active immunization of female mice with LDH-X in those animals with the highest levels of humoral immunity (Erickson, Stites & Spielmann, 1975c). This effect was due to humoral immunity rather than to cellular immunity as assayed by a lymphocyte stimulation test. However, these data did not indicate the time of action of the antibody. Our previous results (Erickson *et al.*, 1975a, b, c) suggested that the antifertility effects of immunization against LDH-X were on the gametes rather than on the preimplantation embryos, and this finding is also suggested by our present investigation, since we could not detect any LDH-X or LDH-X-like receptors in or on preimplantation mouse embryos.

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