Insulin Antibodies Retard and Insulin Accelerates Growth and Differentiation in Early Embryos

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SUMMARY

The physiologic function of insulin in early embryonic life is unknown. We have shown that insulin is present in unfertilized eggs and in chick embryos at 2–3 days of development, even before the emergence of the endocrine pancreas. To define insulin's role, we exposed 2-day-old chick embryos to anti-insulin antibodies and followed their development up to day 5. Antibody-treated embryos had a higher rate of growth retardation and death by days 3–5 of embryogenesis, compared with controls. Among the survivors, biochemical maturation was delayed at days 4 and 5; weight, protein, total creatine kinase activity, and creatine kinase-MB were decreased in antibody-treated embryos.

By contrast, insulin (50 ng/embryo) administered to 2-day-old embryos yielded nearly symmetrical stimulatory results. These findings suggest that endogenous insulin plays a probable physiologic role regulating growth and differentiation in early embryos. In addition, the findings provide some clues to a possible function for insulin produced outside the organism's own beta cells. DIABETES 1985; 34:1063–67.

Pancreatic insulin and its secretion do not appear in chick embryos until 3.5–4 days of embryogenesis.[1] Nevertheless, insulin is present in unfertilized chicken eggs[2,3] as well as in chick embryos at days 2 and 3 of development.[3] The physiologic function of this embryonic insulin is unknown. (It is extrapancreatic in origin, at least in the sense of not being produced in the embryo's own pancreas.) The chick embryo provides many advantages for examining this problem because of its well-studied pattern of development[4] and its isolation from maternal influences.

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To evaluate embryo maturation biochemically, we studied creatine kinase activity, which is found in abundance in skeletal and heart muscle and also occurs in brain and other tissues.[5] The change in the relative proportion of isozymes CK-BB (ubiquitous or embryonic) and CK-MB (more differentiated tissue form) is developmentally regulated[5] and inducible by insulin[6] and, therefore, a good marker for differentiation.

In the present study, we show that embryos at a prepancreatic stage (day 2) exposed to anti-insulin antibodies have an increased fatality rate. In the survivors, morphologic and biochemical development are retarded. Furthermore, in embryos at the same stage, insulin at low doses induces stimulatory changes opposite to those seen with anti-insulin antibodies.

MATERIALS AND METHODS

Chick embryos and injection procedure. Fertilized White Leghorn eggs were incubated at 38 ± 0.5°C and 70–80% relative humidity. At 2 days of development, a window was drilled in the shell over each embryo and embryos that were between developmental stages 10 and 14 of the Hamburger and Hamilton classification[4] were selected for study. The injection technique has been described elsewhere;[7] essentially, the volume of antibody (100 μl) or insulin (50 μl) was applied drop-wise onto the chorioallantoic membrane covering the entire embryo surface, and left in place for the duration of the experiment. Although this delivery system may appear to be subject to imprecision, we have obtained very reproducible effects, and it allows near-normal development in the majority of control embryos.

Antibodies, peptides, and controls. Anti-insulin antiserum (lot 624, guinea pig antipork insulin antiserum, Department of Pharmacology, Indiana University, Indianapolis, Indiana) and control serum (normal guinea pig serum, Miles Research Laboratories, Elkhart, Indiana) were fractionated before administration. Whole serum was filtered on a protein-A Sepharose column; the eluted IgG was used for experiments at a final protein concentration of 4.0–4.8 mg/ml. The purified antibody had an insulin binding capacity of approximately

15 μg/ml. In general, polyclonal antiporcine insulin antibodies recognize porcine, human, and bovine insulin very well, chicken insulin well, proinsulin less well, and they do not recognize insulin-like growth factors (IGFs) or other tissue growth factors.[3,8] Under radioimmunoassay conditions, we tested the ability of different peptides to displace a porcine insulin tracer bound to antibody #624. For it to compete as well as 10 pg chicken insulin we needed 100,000 pg of IGF-II (MSA, Sigma, St. Louis, Missouri).

In three additional experiments, insulin antiserum was further purified by affinity chromatography. Antiserum (2 ml) was treated with 40% ammonium sulfate; the precipitate was reconstituted in 2 ml barbital buffer (pH 8.8) containing 0.2% BSA, and applied to a column of Sepharose-4B to which porcine insulin had been covalently attached. After 3 days at 4°C the column was washed with PBS (pH 8) and the antibody eluted with HCl 1 N and 3 N containing 0.2% BSA. The eluate was neutralized, dialyzed against phosphate buffer, lyophilized, and reconstituted in distilled water. Despite having most of the protein, the flow-through did not bind ^{125}I-insulin under radioimmunoassay conditions, while the eluate showed a good recovery of the antibody.

Other antisera included antibody against epidermal growth factor, nerve growth factor (Collaborative Research, Waltham, Massachusetts), somatostatin (kind gift of Dr. Dorothy T. Krieger), substance P (kind gift of Dr. Susan E. Leeman), and TRH (kind gift of Dr. Krieger). These were purified by precipitation with 40% ammonium sulfate and dialysis before use. A comparable concentration (8–10 mg/ml) of fractionated normal guinea pig serum was used as control. Porcine insulin (lot ODY 44C, Elanco Products Inc.) was kept in stock solution of 1 mg/ml in 0.01 N HCl. Further dilutions in 0.9% NaCl were prepared before the injections. Analytical grade HCl appropriately diluted was used as control for insulin.

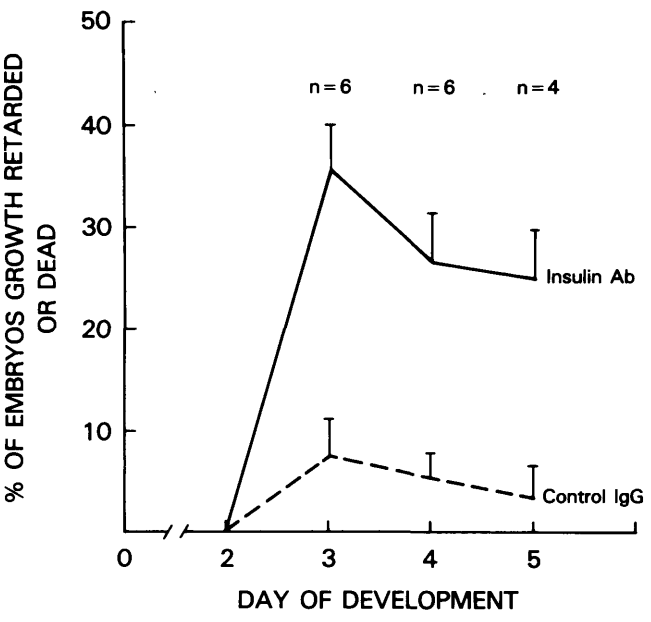

FIGURE 1. Effects of anti-insulin antibody on overall morphologic development. One hundred microliters of protein-A-purified anti-insulin antibody or control IgG was applied to embryos at day 2. The percentage of embryos dead or growth-retarded (stage of development less than that appropriate for age according to Hamburger and Hamilton[4]) was scored after 3, 4, and 5 days of incubation. Differences between antibody-treated and control groups were significant ($P < 0.01$) at all ages.

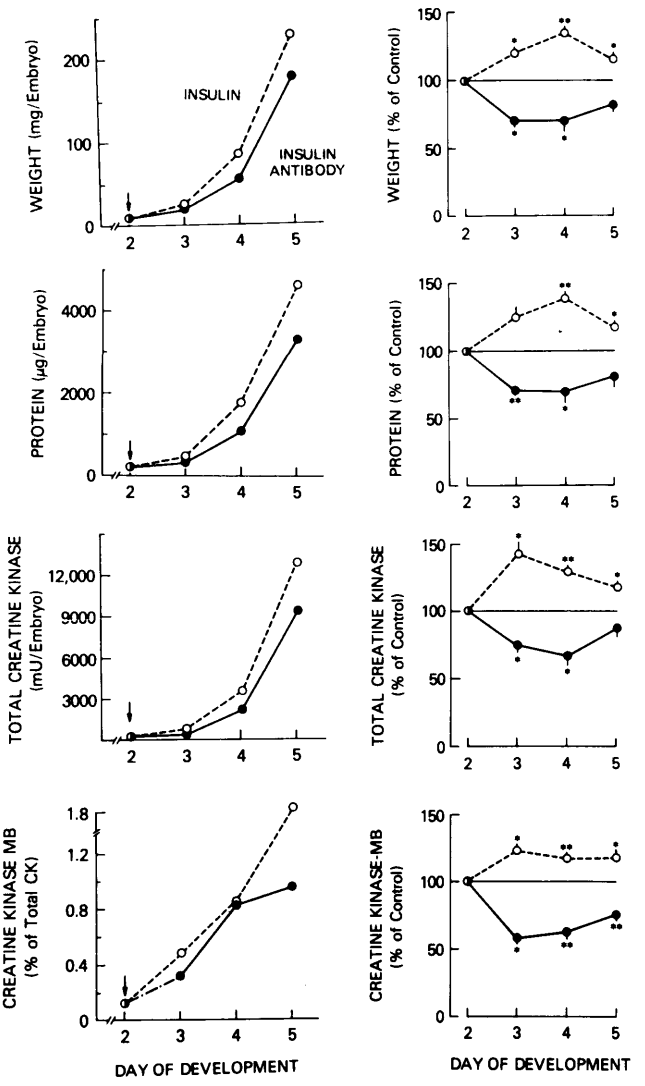

FIGURE 2. Biochemical effects of insulin (50 ng/embryo) and anti-insulin antibody (400 μg/embryo). Materials were applied to embryos at day 2 in parallel with control injected groups (HCl was the control for insulin and normal IgG the control for anti-insulin antibody). Live embryos at days 3, 4, and 5 were homogenized and biochemical parameters were measured. Results on left panels are the means of 3–5 experiments at each age. The natural variation in growth among embryos from different experiments precludes further analysis of data expressed directly as per embryo. A more appropriate analysis is depicted in the right panels where each treated group is compared with respective controls (100%). Differences in insulin-treated versus HCl as well as anti-insulin antibody-treated versus IgG were analyzed by Student's *t*-test for paired samples. * $P < 0.05$, ** $P < 0.005$.

Morphologic evaluation of embryos. Each embryo was evaluated under a stereomicroscope after 3, 4, and 5 days of development. When the heartbeat was not detected, the embryo was considered dead and was discarded. The live embryos were examined to assess the stage of development according to the classification of Hamburger and Hamilton.[4] The head, limb buds, tail, and extraembryonic vessels are the main morphologic features used to classify the embryo at these stages. (At this age, somites are not useful as developmental markers.) Embryos underdeveloped for their age (below stage 18 at day 3, below stage 22 at day 4, and

TABLE 1
Embryos at day 4 of development treated with affinity-purified anti-insulin antibody at day 2

Experiment	Weight (% of control)	Protein (% of control)	CK-MB (% of control)
1	94.5	84.3	75.5
2	70.5	69.4	68.5
3	76.9	74.8	50.5
Mean ± SEM	81 ± 5	76 ± 3	65 ± 6
P-value	<0.05	<0.005	<0.005

below stage 26 at day 5) were recorded. All live embryos, whether macroscopically normal or retarded, were dissected from the membranes, washed in saline, and kept at −70°C for biochemical determinations.

Biochemical determinations in survivors. The surviving embryos in each group were weighed and homogenized in 4 vol of cold Tris-HCl (50 nM, pH 7.5, twice for 30 s each) with an Ultra-Turrax homogenizer. Each homogenate was centrifuged at 1000 × g for 10 min at 4°C, and the supernatant was used for protein and creatine kinase determinations. Protein concentration was measured by the method of Bradford.[9] Creatine kinase activity was measured spectrophotometrically at 340 nm (centrifugal Analyzer Centrifichem 400) after addition to CK-NAC-activated reagent (Boehringer-Mannheim, Indianapolis, Indiana) and 0.12 nM diadenosine pentaphosphate (myokinase inhibitor). The separation of BB and MB isoenzymes was carried out by continuous salt gradient elution from a 0.9 × 9-cm DEAE-Sepharose CL-6B column.[10] Samples of embryo homogenates containing approximately 6000 mU of creatine kinase (0.7–1.4 ml vol) were filtered on a column and 90 fractions eluted with a linear 0–250 mM NaCl gradient (in Tris-HCl, 50 mM, pH 7.5). Creatine kinase activity was measured in individual fractions, and peaks corresponding to MB isozyme (elution at 55 mM Na^+) and BB isozyme (elution at 100 mM Na^+) were calculated. DNA and RNA contents were measured with ethidium bromide.[11]

RESULTS

Abnormal development and death rate after antibody treatment. The adminstration of protein-A-purified anti-insulin antibodies to day-2 embryos caused remarkably higher mortality than did normal IgG and, in survivors, produced macroscopic growth retardation at days 3, 4, and 5 (Figure 1). Among the survivors, the antibody-treated embryos showed a reduction in weight, total protein, and total creatine kinase by days 3 and 4 of development (Figure 2). The proportion of the isozyme creatine kinase-MB relative to total enzyme activity was reduced in treated embryos at all ages studied (days 3–5). DNA and RNA contents were measured in live embryos at day 4. Prior treatment with anti-insulin antibody decreased both the content of DNA (to 73 ± 7% of control group, $P < 0.05$) and RNA (to 67 ± 7% of control group, $P < 0.02$).

Anti-insulin antibodies, purified further by affinity chromatography with insulin linked to Sepharose, were also applied to day-2 embryos. Again, we observed at day 4 the decrease in weight, total protein and creatine kinase-MB isozyme (Table 1). (Mortality was reduced to control levels possibly due to loss of antibody titer during purification.) The flow-through of the affinity column, which contained the bulk of the protein but none of the antibody, was without deleterious effect on the embryos.

To show that the effects were specific to anti-insulin antibodies, antisera to several growth factors and unrelated hormones were injected into eggs. There were no significant deleterious effects on growth between days 2 and 4 of embryogenesis when compared with effects of normal serum (Table 2).

Effects of insulin. Application of purified insulin to day-2 embryos produced biochemical changes opposite to those elicited by the anti-insulin antibodies (Figure 2). Embryos treated with 50 ng of insulin had increased weight, protein, total creatine kinase, and creatine kinase-MB at days 3, 4, and 5 of development. The effects of insulin were dose-dependent in the range of 50–100 ng/embryo (Figure 3).

DISCUSSION

To elucidate the role of embryonic insulin, we exposed embryos at early stages to anti-insulin antibodies and to small doses of insulin. Development by morphologic and biochemical criteria was stopped or retarded by the antibodies and "mirror-like" stimulatory changes were observed with insulin. Thus, we raise the possibility that in young chick embryos insulin, or another peptide that is reactive with anti-insulin antibodies (but probably not IGFs, which react extremely weakly with anti-insulin antibodies), is required and contributes to some aspects of normal development. The insulin injection experiments are "pharmacologic" while the anti-insulin antibody injections provide "physiologic" insights. That the two sets of experiments yielded near-exact results is consistent with our suggestion that there is an endogenous insulin-related material that regulates development pathways. It should be emphasized that we do not yet know whether the effects we observe are exerted directly on the embryo's own tissue or at extraembryonic sites in the egg. While effects on extraembryonic membranes are possible, we do not think it is likely since the chorioallantoic membrane repeatedly had the lowest levels of binding of ^{125}I-insulin, ^{125}I-IGF I, and ^{125}I-IGF II of any embryonic tissue that we tested

TABLE 2
Embryos at day 4 of development treated with other antibodies at day 2

Antisera	Weight	Protein	Total CK	CK-MB
Ab-EGF				
Expt. 1	116	109	113	111
Expt. 2	108	106	89	94
Ab-NGF				
Expt. 1	117	117	120	NT
Expt. 2	138	134	156	131
Ab-Somatostatin				
Expt. 1	110	115	85	NT
Expt. 2	154	160	184	117
Ab-Substance P				
Expt. 1	104	100	89	NT
Expt. 2	131	129	157	68

Effects of antisera precipitated with 40% ammonium sulfate are given as percentages of control-injected (non-immune serum) embryos. In all cases, mortality and growth retardation occurred in less than 10% of embryos. NT = not tested.

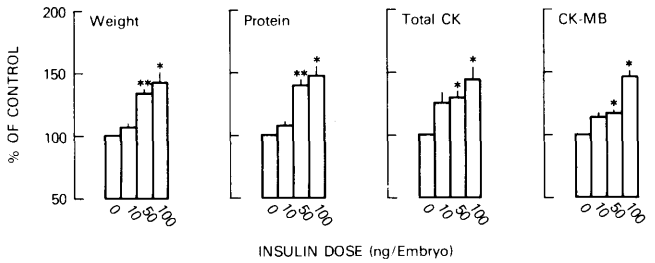

FIGURE 3. Effects of insulin applied on embryos at day 2 and evaluated at day 4. Values are represented as percent obtained in control embryos treated with diluted HCl alone. Differences with respect to "zero" dose were significant at the level of * $P < 0.05$, and ** $P < 0.002$.

(unpublished data). Similarly, it is not known whether the effects we observe are primarily on "metabolic" pathways (e.g., glucose, lipid, and amino acid metabolism) and secondarily on the disruption of growth and development, or whether the effects are directly on "growth" pathways. In the case of the latter, the effects may be directly on development processes or largely on the inhibition of formation or release of growth factors such as the IGFs. In older mammals, production of IGFs and expression of IGF receptors in some circumstances are insulin-dependent processes.[12] While the pathway is uncertain, it should be emphasized again that the primary molecular target of the antibody is insulin or a very close relative. In preliminary experiments, we have found that the deleterious effects of anti-insulin antibody are still demonstrable when we inject 50 ng/embryo of IGF-I (AmGen) together with the anti-insulin antibody, whereas added insulin abolishes most of the deleterious effects of the antibody (results not shown).

The prevailing feeling among developmental biologists seems to be that hormonal influences on embryonic development start late,[13] largely because the usual focus of hormone production is the endocrine gland and these glands appear at relatively advanced states of differentiation. The finding that insulin, presumably derived from sources outside the embryo's beta cells, plays an important role in the early embryo raises the possibility that nontraditional sources of hormone may be physiologically significant (in addition to any contribution from the emerging beta cells). Interestingly, prothoracicotrophic hormone, the brain peptide of insects that drives metamorphosis, has substantial structural similarity to insulin and insulin-like growth factor I,[14] although it does not react with anti-insulin antibodies. Among vertebrates, insulin stimulates differentiation in several embryonic cell types in culture, including myoblasts, which express creatine kinase-MB isozyme,[6] neurons,[15] and whole oocytes.[16,17] Certainly, at later stages of embryogenesis insulin has been shown to have typical metabolic[18,19] and growth-promoting effects.[20,21] Insulin- (and proinsulin-) related molecules can act through insulin receptors to produce both metabolic and growth effects, or, less effectively, through type 1 receptors for IGFs. The present study sheds no light on which receptors are involved. We have found that IGF-type 1 receptors are the dominant binders for insulin, at least in brain, in embryos at early stages.[22] Autoradiographic studies with a detailed search for insulin and IGF receptors at early stages of embryogenesis, and the use of receptor antibodies with narrow specificity,* will be required to clarify which receptors mediate what appear to be in vivo physiologic effects of insulin in early development. To elucidate the role of insulin and the possible effects of antibodies at early stages may also help in the understanding of any additional mechanisms for the first trimester complications observed in infants of mothers with type I diabetes.

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REFERENCES

[1] Dieterlen-Lievre, F., and Beaupain, D.: Immunocytological study of endocrine pancreas ontogeny in the chick embryo: normal development and pancreatic potentialities in the early splachnopleura. *In* The Evolution of Pancreatic Islets. Adenasanya, T., Grillo, I., Leibson, L., and Epple, A., Eds. Oxford, Pergamon Press, 1976:37–50.

[2] Trenkle, A., and Hopkins, K.: Immunological investigation of an insulin-like substance in the chicken egg. Gen. Comp. Endocrinol. 1971; 16:493–97.

[3] De Pablo, F., Roth, J., Hernandez, E., and Pruss, R. B.: Insulin is present in chicken eggs and early chick embryos. Endocrinology 1982; 111:1909–16.

[4] Hamburger, V., and Hamilton, H. L.: A series of normal stages in the development of the chick embryo. J. Morphol. 1951; 88:49–92.

[5] Eppenberger, H. M., Perriard, J. C., and Wallimann, T.: Analysis of creatine kinase isozymes during muscle differentiation. *In* Isozymes: Current Topics in Biological and Medical Research. Rattazzi, M. C., Scandalios, J. G., and Whitt, G. S., Eds. New York, Alan R. Liss, Inc., 1983:19–38.

[6] Ewton, D. C., and Florini, J. R.: Effects of somatomedins and insulin on myoblast differentiation in vitro. Dev. Biol. 1981; 86:31–39.

[7] De Pablo, F., Hernandez, E., Collia, F., and Gomez, J. A.: Untoward effects of pharmacological doses of insulin in early chick embryos: through which receptors are they mediated? Diabetologia 1985; 28:308–13.

[8] Rosenzweig, J. L., Lesniak, M. A., Samuels, B. E., Yip, C. C., Zimmerman, A. E., and Roth, J.: Insulin in extrapancreatic tissues of guinea pig differs markedly from the insulin in their plasma and pancreas. Trans. Assoc. Am. Physicians 1980; 93:263–78.

[9] Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 1976; 72:248–54.

[10] Yasmineh, W. G., and Hanson, N. Q.: Electrophoresis on cellulose acetate and chromatography on DEAE-Sephadex A-50 compared in the estimation of creatine kinase isoenzymes. Clin. Chem. 1975; 21:381–86.

[11] Boer, G. J.: A simplified microassay of DNA and RNA using ethidium bromide. Anal. Biochem. 1975; 65:225–31.

[12] Nissley, S. P., and Rechler, M. M.: Insulin-like growth factors: biosynthesis, receptors and carrier proteins. *In* Hormonal Proteins and Peptides. Li, C. H., Ed. New York, Academic Press, 1985:127–203.

[13] Tata, J. R.: The action of growth and developmental hormones: evolutionary aspects. *In* Biological Regulation and Development. Goldberger, R. F., and Yamamoto, K. R., Eds. New York, Plenum Publishing, 1984:1–58.

[14] Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Ishizaki, H., Mizoguchi, A., Fujiwara, Y., and Suzuki, A.: Amino-terminal amino acid sequence of the silkworm prothoracicotropic hormone: homology with insulin. Science 1984; 226:1344–45.

[15] Puro, D. G., and Agardh, E.: Insulin-mediated regulation of neuronal maturation. Science 1984; 225:1170–72.

[16] El-Etr, M., Schorderet-Slatkine, S., and Baulieu, E. E.: Meiotic maturation in "Xenopus laevis" oocytes initiated by insulin. Science 1979; 205:1397–99.

[17] Maller, J. L., and Koontz, J. W.: A study of the induction of cell division in amphibian oocytes by insulin. Dev. Biol. 1981; 85:309–16.

*Preliminary experiments with polyclonal anti-insulin receptor antibody have reproduced the effects of anti-insulin antibodies.

[18] Zwilling, E.: Insulin hypoglycemia in chick embryos. Proc. Soc. Exp. Biol. Med. 1948; 67:192.

[19] Leibson, L., Bondareva, V., and Soltitskaya, L.: The secretion and the role of insulin in chick embryos and chickens. *In* The Evolution of Pancreatic Islets. Oxford, Pergamon Press, 1976:69–79.

[20] Susa, J. B., Neave, C., Sehgal, P., Singer, D. B., Zeller, W. P., and Schwartz, R.: Chronic hyperinsulinemia in the fetal rhesus monkey: effects of physiologic hyperinsulinemia on fetal growth and composition. Diabetes 1984; 33:656–60.

[21] Angervall, L., Karlsoon, K., and Martinsson, A.: Effects on the rat fetuses of intrauterine injections of insulin. Diabetologia 1981; 20:558–62.

[22] Bassas, L. L., De Pablo, F., Lesniak, M. A., and Roth, J.: Receptors that prefer insulin-like growth factors antedate the insulin receptor during organogenesis of the chick. Abstract. J. Steroid Biochem. 1984; 20:1423.