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Insulin
newborn infants
glucose tolerance
diabetes
anti-insulin antibodies
gamma-globulin

The Effect of Endogenous Antibody on Insulin-assay in the Newborn Infants of Diabetic Mothers

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Extract

The immunoassay of insulin in blood of infants born to diabetic mothers is complicated by the presence of anti-insulin antibodies.

Twelve infants born to diabetic mothers receiving insulin up to the time of delivery (Group 1) and two born to diabetic mothers maintained by dietary restrictions without insulin (Group 2) were studied. Four infants born to normal mothers served as controls (Group 3). The range of values in cord blood of infants of Group 1 was 80–1000 (median 550) (method of Hales and Randle) and 33 to 72.5 % (median 53.8) of the radioactivity was bound to plasma proteins. In one infant in Group 1, maximum amount of the radioactivity (42.3 %) from labeled insulin (43 %) was bound to a γ-globulin (0 % in Groups 2 and 3). In one infant in Groups 2 and 3 maximum amount of radioactivity was in the α-globulin fraction (44.1 and 35.3 % respectively).

At 120 minutes after birth, glucose, 0.5 gm/kg body weight, was infused quickly into the umbilical vein of 6 patients in Group 1 and the two in Group 2. In the former, insulin levels rose significantly, 1- to 4-fold, despite the elevated level at commencement of infusion; in the latter, the insulin levels rose 4-fold in one case and 18-fold in the second. The amount of anti-insulin antibody present in serum of infants in Group 1 did not change significantly during the first 240 minutes after birth; neither was it affected by the administration of glucose and the subsequent appearance of endogenous insulin.

Speculation

Although the infant born to a diabetic mother has elevated levels of insulin and circulating anti-insulin antibodies, his ability to release insulin following a glucose challenge appears unimpaired. This combination of abnormalities suggests an alteration in glucose homeostasis, but fails to explain the hypoglycemia commonly seen in such infants, unless the anti-insulin antibodies do not interfere with the hypoglycemic effects of insulin.

Introduction

The development of immuno-chemical methods for the assay of insulin following the work of Yalow and Berson [1960] has made readily available suitable micro-techniques which are specific for the insulin molecule. The present paper outlines a study to investigate the production of insulin in the newborn infants of diabetic mothers and to confirm and extend the work previously reported by Baird and Farquhar [1962]. Some of the problems encountered, which were due to interference in the immuno-chemical assay procedures by endogenous maternal antibodies to insulin, are discussed.

Patients and Methods

The infants described were born at the Simpson Memorial Maternity Pavilion to fourteen diabetic women of whom twelve had been treated with insulin up to the time of delivery (table I). One other had received insulin over a four-year period but had had none in the eight years prior to this birth, having had dietary treatment and chlorpropamide. Another had been treated with diet alone. The mothers of the four control infants were judged to be normal. These infants were born at full-term and were of normal birth weight. The mothers had no family history of diabetes mellitus, and their previous obstetric records were unremarkable. Each had had an average of twelve urine tests from first reporting for antenatal care up to the actual delivery, and none had glycosuria. Under these circumstances further glucose tolerance testing was not undertaken.

The plasma insulin was studied only in the cord blood of some babies, but in others this was accompanied by an investigation of the plasma insulin response to the injection of glucose. When this was to be done the baby was first given a placental transfusion in order to ensure an adequate blood volume. The method of injecting glucose into and of taking blood samples from the umbilical vein was similar to that previously used (BAIRD and FARQUHAR [1962]) but the tip of the catheter was positioned at less than 7.5 cm from the anterior abdominal wall in most cases when blood was freely obtained.

Glucose was injected at exactly two hours after birth, and plasma insulin[1] was determined before and so far as this was possible on seven occasions after the glucose had been given. The dose of glucose was again calculated as 0.5 g per kg body weight, and it was given quickly as a 20 % solution. All infants were handled as little as possible and were nursed comfortably at an environmental temperature of approximately 32° C.

Plasma insulin was measured by immuno-assay techniques. These methods use a specific antiserum for sampling mixtures of insulin and radioactive insulin

[1] The term 'insulin' here means immunologically-reactive insulin under the conditions of the method used.

Table I. Clinical details

Case No.	Age of mother (years)	Duration of mother's diabetes (years)	Treatment of mother	Mode of delivery	Maturity of baby (days)	Weight of baby (grams)
Normals						
J.4400	29	—	—	vaginal	277	3400
J.4491	22	—	—	vaginal	280	3430
J.4658	22	—	—	vaginal	276	3600
J.4688	29	—	—	vaginal	275	3690
Diabetics						
296	22	9	diet+insulin	vaginal	257	3690
297	33	12	diet+insulin	caesarean section	231	2810
298	30	16	diet+insulin	caesarean section	254	3570
299	26	12	diet+insulin	caesarean section	244	3200
300	23	2	diet+insulin	caesarean section	252	2880
302	27	17	diet+insulin	caesarean section	255	4110
303	28	in pregnancy	diet+insulin	caesarean section	257	3230
307	33	17	diet+insulin	vaginal	258	3690
309	24	in pregnancy	diet	vaginal	248	3840
310	28	13	diet+insulin 1953–1957 then diet+ chlorpropamide	caesarean section	233	2060
311	23	16	diet+insulin	caesarean section	245	2230
313	19	1	diet+insulin	caesarean section	256	4680
314	33	29	diet+insulin	vaginal	247	2040
315	31	5	diet+insulin	caesarean section	247	2610

under conditions where the proportion of antibody-bound radioactivity is related to the non-radioactive insulin concentration.

The procedures used were based on those of YALOW and BERSON [1960], in which the antibody-bound insulin and the free insulin were separated by paper chromatography using 0.072M barbitone buffer, pH 8.6 as solvent, and of HALES and RANDLE [1963] in which cellulose acetate filters were used to retain the bound insulin. This method is fully described in The Radiochemical Centre Data Sheet 5581 [1965].

The ^{125}I-insulin was obtained from The Radiochemical Centre, Amersham, Bucks, England, and had a specific activity of not less than 50 microcuries per microgram.

The anti-insulin serum and the insulin binding reagent, consisting of the antibody precipitate from the first stage of Method C of HALES and RANDLE [1963], were prepared at The Wellcome Research Laboratories. These antisera react under the conditions described with similar sensitivity to ox, human and pig insulins. The final dilution of the anti-insulin sera was 1/48,000.

The sensitivity of the assay systems was 0.4 microunits insulin, and the standard deviation over the concentration range 0.4 to 10 microunits insulin was 3 to 4 % of the mean.

The binding of ^{125}I-insulin by plasma proteins was determined by the chromatographic separation used in the YALOW and BERSON [1960] immuno-assay technique.

Results

a) Recognition of Interference in the Assay

The insulin levels in the plasma of cord blood assayed by the HALES and RANDLE [1963] method are shown in table II and are in general very much higher, with the exceptions described below, than expected from previous work using the rat diaphragm method of insulin assay (BAIRD and FARQUHAR [1962]). To test the validity of these findings insulin assay by the YALOW and BERSON [1960] technique was carried out in some cases but indicated negligible amounts of insulin present. This method differs essentially from that of HALES and RANDLE [1963] only in the way by which insulin bound to the added antibody is separated from the unbound 'free' insulin.

Chromatographic separation as used in the YALOW and BERSON [1960] method showed that considerable amounts of radioactive insulin migrated on filter-paper strips with the plasma proteins of infants of insulin-treated diabetic mothers, even in the absence of added anti-insulin serum. Cases 309 and 310 behaved differently, the mother of the first never having had insulin while that of the second had had none in recent years (table II).

b) Localisation of the Interference

The electrophoretic separation of plasma proteins on cellulose acetate strips in 0.04 M barbitone buffer at pH 8.6 following the incubation at 4°C at pH 7.4 of 0.2 ml of plasma with 250 micro-micrograms of ^{125}I-insulin for four days indicated that where binding of ^{125}I-insulin in plasma was high the radioactivity was found in the gammaglobulin and inter-beta-gamma zones. Where the binding was low the radioactivity was mainly in the alpha globulin zones (table III) and probably represents denatured insulin. These findings are in keeping with those of PROUT *et al.* [1963].

On precipitation of the globulins by the addition of sodium sulphite to a final concentration of 22 per cent, 38 per cent of the radioactivity remained in the precipitate after solution and reprecipitation in the case of the infant of the diabetic mother treated with insulin, 18 per cent in the infant of the non-insulin treated diabetic mother, and 22 per cent in the infant of the non-diabetic mother. The precipitates redissolved in an 0.85 per cent solution of sodium chloride were subjected to zone electrophoresis on cellulose acetate at pH 8.6. The fractions representing gamma and beta globulins were the only ones present in significant amounts.

From these findings it was concluded that insulin was bound to a protein or proteins present in the gamma and inter-beta-gamma fractions of plasma and that this protein was similar or identical to the antibody produced in diabetics receiving insulin therapeutically for a period of time.

c) Dilution of Interference

An attempt was made to overcome the effects of the endogenous antibody on the assay of insulin by dilution of the plasma to give final dilutions of between 1 in 3 and 1 in 30. Dilution beyond this was not considered practicable because of loss of sensitivity in the assay method. Where reasonable agreement was achieved between the HALES and RANDLE method and that of YALOW and BERSON, dilution had reduced the effect of the endogenous antibody to negligible proportions. The effect could not be eliminated in other cases (table IV). As the interference in the immuno-assay techniques is due to a specific antibody and not to a non-specific antagonist, the effect can at best be only minimized and not completely removed as the antibody retains a proportionate activity at any dilution.

d) Plasma Insulin Response to Injected Glucose

It was thus possible to follow the changes in insulin levels after the administration of intravenous glucose

Table II. Cord blood plasma 'insulin' levels and ^{125}I-insulin binding (final plasma dilution 1 in 3)

Case No.	Insulin μU/ml (Hales and Randle method)	% radioactivity bound by plasma proteins (paper chromatography)
Infants of diabetics		
296	162	65.2
297	>>1000	53.8
298	410	33.0
299	904	50.8
300	>>>1000	46.5
302	150	42.3
303	670	62.8
307	>1000	59.1
311	90	44.0
313	>1000	53.1
314	550	72.5
315	80	—
309	24	19.4
310	20	13.2
Normal infants		
J.4400	21	16.4
J.4491	0	—
J.4658	8	15.6
J.4688	61	11.8

Table III. Electrophoretic separation of insulin-binding proteins
% radioactivity bound to proteins

Protein fraction	Baby 299 (mother diabetic, insulin-treated)	Baby 310 (mother diabetic, not on insulin)	Normal baby (mother not diabetic)
γ-globulin	42.3	0	0
Fibrinogen	30.5	2.0	3.9
β-globulin	3.3	10.7	10.1
a_2-globulin	12.8	29.5	30.4
a_1-globulin	11.1	44.1	35.3
Albumin	0	13.7	20.3

to the infants of diabetic mothers. While these values do not necessarily represent the absolute amounts of insulin in the plasma, as the endogenous antibody had still some effect in most cases, it was possible to show that the infants of the diabetic mothers responded to an intravenous glucose load by sharp increments in insulin levels almost immediately (table 4) and that the insulin levels with one exception fell rapidly to values near the pre-glucose levels. In those babies, 309 and 310, whose mothers had had no recent insulin therapy and whose plasma insulin binding capacity was correspondingly low, the rise in plasma insulin level was clear.

Table IV. Insulin levels in plasma diluted to reduce interference (newborn infants of diabetic mothers)

Specimen	Plasma insulin in microunits/ml of plasma															
Baby serial number	296		297		298		299		302		307		309		310	
Final plasma dilution	1 in 3		1 in 15		1 in 30		1 in 15		1 in 15		1 in 15		1 in 6		1 in 6	
Method[1]	H	Y	H	Y	H	Y	H	Y	H	Y	H	Y	H	Y	H	Y
Time after birth																
Maternal	—	—	—	—	—	—	—	—	—	—	490	115	6	0	0	0
Cord 0 minutes	162	—	1525	17	250	—	60	—	165	135	1025	155	14	10	18	26
120 minutes	149	—	590	13	70	—	95	—	80	85	440	110	10	0	11	0
Glucose	0.5 g/kg given quickly into umbilical vein at 120 minutes															
122 minutes	190	—	—	—	200	—	260	—	390	185	1060	165	180	200	44	30
125 minutes	194	—	775	19	140	—	215	—	275	145	735	140	102	156	33	40
130 minutes	191	—	605	16	70	—	125	—	185	120	625	150	44	48	—	—
140 minutes	204	—	645	13	70	—	130	—	—	—	630	120	24	19	—	—
150 minutes	220	—	415	14	30	—	125	—	100	90	450	100	10	12	11	0
180 minutes	220	—	425	12	0	—	105	—	50	65	410	90	14	13	—	—
240 minutes	202	—	—	—	0	—	80	—	65	65	270	80	24	19	—	—

[1] H = HALES and RANDLE; Y = YALOW and BERSON.

As exemplified in table V, the changes in plasma insulin levels following glucose could not be explained by changes in the binding of radioactivity by the endogenous antibody.

Table V. Insulin levels and the binding of radioactivity by endogenous antibody in the 4 hours after birth e.g. Baby 307

Specimen time after birth	% radioactivity bound by plasma proteins (paper chromatography)	Insulin μU/ml (HALES and RANDLE method)
Maternal	39.7	490
Cord	35.4	1025
120	36.5	440
122	33.5	1060
125	32.6	735
130	35.3	625
140	38.4	630
150	36.9	450
180	40.2	410
240	39.3	270

Discussion

The presence of antibodies in the plasma of insulin-treated diabetics has been recognized for some time (LERMAN [1944]; LOWELL [1944]), and recently SPELLACY and GOETZ [1963] demonstrated that non-precipitating insulin-binding antibodies were found in newborn infants whose diabetic mothers had received insulin therapy. These antibodies were believed to originate in the mother.

Plasma insulin determinations by immuno-assay procedures in insulin-treated adults are made difficult by the presence of endogenous insulin antibodies (YALOW and BERSON [1961]). The presence of antibody affects the assay in a way which is unique to immuno-assays but the effect upon the apparent values for insulin depends upon which of the variations of the immuno-chemical methods is used. As the present work shows, the method which separates the insulin-antibody complex in the soluble form (YALOW and BERSON [1960]) will give low values for insulin in the presence of endogenous antibody, while the method of HALES and RANDLE [1963], and also that of MORGAN and LAZAROW [1963], which separate the insulin-antibody complex in an insoluble form, gives high values for insulin in the presence of endogenous antibody. Possibly the interference by endogenous antibody could account for the high values found by immuno-assay techniques in those infants of diabetic mothers studied by STIMMLER, BRAZIE and O'BRIEN [1964] as pointed out by KLINK and ESTRICH [1964].

YALOW and BERSON [1961] found that this problem could occasionally be overcome by making use of the great sensitivity of their immuno-assay procedure with human ^{131}I-insulin and by diluting the plasma as much as fifty times. In the present study the apparent high values for plasma insulin could be rationalised by dilution of the plasma, though the sensitivity of the method limited the extent of dilution. The plasma of those infants whose mothers had had no or no recent insulin gave comparable results with diluted and undiluted specimens. In most cases the plasma antibodies were still capable of binding considerable amounts of ^{125}I-insulin even at the greatest dilutions used, and in these cases the guinea-pig anti-insulin serum added for the assay was not able effectively to reduce the amount of radioactivity which was bound to the plasma antibody. The importance of establishing the extent to which plasma gamma globulins will bind ^{125}I-insulin before accepting values for plasma insulin obtained by immuno-assay seems clear. This is necessary even with diluted plasma.

Preliminary extraction of plasma with acid-alcohol has been used (GRODSKY and FORSHAM [1960]) to allow the determination of insulin in plasma from adult diabetics with circulating non-precipitating insulin antibodies. The limitations imposed on the size of the samples which could be taken from newborn infants prevented this technique from being used effectively in the present study. This same consideration also applied to the method of separation of antibody-bound insulin from unbound 'free' insulin by gel filtration (MANIPOL and SPITZY [1962]). Ultra-centrifugation has also been used for this separation (YALOW and BERSON [1961]). An increase in the sensitivity of the method of assay together with greater dilution of plasma could offset the effects of the interference but would certainly not eliminate it in many cases.

Allowing that the effects of the endogenous antibody have not been entirely removed, the present study indicates that the infant of the diabetic mother responds to an intravenous glucose load by producing immediate and large increases in plasma insulin similar to those found previously (BAIRD and FARQUHAR [1962]). Given that the insulin levels found cannot be explained by increased binding capacity following the glucose load, and that the maternal antibody to animal insulin seems to have low affinity for human insulin (YALOW and BERSON [1961]), it does appear that the infant of the diabetic mother responds to a glucose load by release of insulin. This is also the experience of JORGENSEN, DECKERT, PEDERSEN and PEDERSEN [1966] who, faced with the same problem of antibody inter-

ference, found a sharp rise in insulin levels after an intravenous glucose load. In contrast to the present study they gave the glucose at a variable time after birth and chose to look for the insulin response five minutes afterward, whereas the present study suggests that the peak may already have been passed at this time. The fasting insulin levels in the two infants of non-insulin-treated diabetics in this series are low, while in the two described by JØRGENSEN *et al.* [1966], they are high. The truth must be decided in a more extensive study, but the present finding is at least consistent with that described by BAIRD and FARQUHAR [1962]. The rapid insulin response to glucose in the babies of non-insulin-treated mothers closely resembles the pattern in those of the insulin-treated group, and is uncomplicated by insulin antibody interference in the method of assay.

Summary

The presence of antibody to exogenous insulin in the plasma of infants newly born to insulin-treated diabetic mothers makes difficult the quantitation of the plasma insulin. Failure to recognise this may cause errors both in measurement and in interpretation.

The effect of maternal antibody may be partly overcome by dilution, and in the present study this method and the investigation of babies born to two mothers who had received little or no previous insulin treatment confirms that newborn infants of diabetic mothers respond very rapidly to an intravenous load of glucose by the release of insulin. Until the problem of endogenous antibody interference with accurate quantitation of plasma insulin by immunochemical methods is overcome, the measurement of such and of the insulin response to injected glucose may require to be confined to those babies whose mothers have not had insulin therapy.

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