ORIGINALS

A New Type of Hypoglycaemia in a Newborn Infant

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Summary. The clinical and immunological findings are described in a female newborn infant who presented with hypoglycemia associated with a high titre of an insulin-binding protein in her serum. The insulin-binding protein belonged to IgG, the light chain of which consisted solely of Kappa-type. The other properties of the insulin-binding protein were very similar to those of insulin antibodies produced by insulin injection, except for a

stronger affinity to human insulin as compared with bovine insulin. The patient's mother had a similar protein in her serum despite no previous history of insulin treatment. It is assumed that the hypoglycemia of this patient might have been due to the presence of the antibody produced in her mother's body against endogenous insulin and transferred to the patient transplacentally.

Many types of hypoglycemia in newborn infants have been described, but the aetiology of hypoglycemia in some patients is still unknown.

Recently, a female baby born in our hospital showed hypoglycemia shortly after birth and despite thorough studies, her hypoglycemia did not belong to the types of hypoglycemia already known.

Clinical Course

A female baby was born by normal delivery at full term (on August 3, 1971) in our hospital. Birth weight was 3459 g. She showed a tremor of the extremities three days after birth and at that time her blood sugar was 38 mg/100 ml with Hoffman's method and her

Fig. 1. The clinical course of the baby. The antibody titre was determined with Sephadex chromatography as described in experimental method and expressed in B/F. The IRI value in this figure was determined with the two antibody method of Morgan and Lazarow with nonextracted serum

Further studies of her insulin dynamics revealed that her serum contained a large amount of an insulin-binding protein, despite no previous history of insulin injection of her or her mother.

In this report, the clinical course and the nature of the insulin-binding substances are described.

serum calcium level was 10.2 mg/100 ml. After that, tremor with hypoglycemia was frequently observed in the fasting state and disappeared with artificial feeding. Electroencephalography performed at intervals was normal.

The results of the oral glucose tolerance test (1.75 g/

kg) and the intravenous tolbutamide test (25 mg/kg) were as follows:

Time after loading of glucose (min.)	0	30	60	90	120	150	180
Blood Sugar (mg/100 ml)	22	62	76	87	100	88	87
Time after loading of tolbutamide (min.)	0	15	30	60	90	120	180
Blood Sugar (mg/100 ml)	84	58	44	47	54	67	62

The responses of her blood sugar to leucine (150 /mg kg, per os), epinephrine (0.1 mg subcutaneously), glucagon (30 µg/kg, i.v.) and insulin (0.1 U/kg, i.v.) were normal. In an arginine test (0.5 g/kg, i.v.), a marked increase in blood sugar and a slightly retarded growth hormone response were observed:

Time after loading of arginine (min.)	-5	0	5	30	60	90	120
Blood Sugar (mg/100 ml)	32	36	82	60	60	44	56
HGH (mug/ml)	11.0	18.5		17.8	37.0	29.5	30.5

Fig. 2. The blood sugar, the free and total insulin levels in the course of the glucose tolerance test (100 g, p.o.) of the mother.

Table 1. Serum IRI and insulin antibody titre

	Serum	Date	Blood sugar (mg/dl)	Free IRI (µU/ml)	Total IRI (µU/ml)	Antibody titre (B/F)
Patient's mother		71. 11. 11.	79	—	—	2.12
		12. 8.	75	9.0	3307	2.26
		72. 3. 3. ^a	75 ^a	25.0	1783	3.68
Patient (baby)		71. 11. 11.	79	3.7	4143	1.82
		12. 1.	81	—	441	1.40
		72. 1. 5. ^a	96	37.0	815	0.85
		2. 5. ^a	75	1.7	171	0.78
		3. 22. ^a	64	17.0	65	0.81
		4. 21. ^a	85	20.4	37	0.54
Grand-mother		72. 3. 27.		19.0	20	0.00

^a: Determined in non-fasting state

B/F: Bound insulin/Free insulin.

The liver function tests and the hemogram were normal. The plasma cortisol levels were 1.5 µg/100 ml and 26.2 µg/100 ml before and 1 h after the intramuscular injection of synthetic ACTH (50 µg/kg). Her immunoglobulin consisted of 730 mg/100 ml of IgG, 75 mg/100 ml of IgM and 20 mg/100 ml of IgA.

The serum immunoreactive insulin (IRI) levels, estimated by the Morgan-Lazarow two antibody method [2] were markedly high fasting and after food (Fig. 1). Her serum showed a high titre of insulin-binding activity and contained a large amount of extractable insulin (Table 1).

The patient's mother, aged 25 years, has had no specific signs or symptoms, but showed a diabetic curve in the oral glucose tolerance test (Fig. 2). Her serum contained a large amount of insulin-binding substance and extractable insulin (Table 1), but no rheumatic factor, thyroglobulin antibody, thyroid microsomal antibody nor anti-nuclear factor; her serum immunoglobulin consisted of 2350 mg/100 ml of IgG, 112 mg/100 ml of IgM and 272 mg/100 ml of IgA.

The baby's hypoglycemia was alleviated, but not completely prevented, with multiple artificial feeding. On December 9, 1971, prednisolone therapy was initi-

ated and the hypoglycemia completely subsided. After a gradual decrease of prednisolone, therapy was suspended on January 10, 1972, without return of the hypoglycaemia.

The insulin-binding substance and the extractable insulin in the baby's serum decreased gradually and her growth was completely normal in the observed period. In contrast to the baby, the mother's serum insulin-binding substance and the extractable insulin have remained at high levels with only slight fluctuations (Table 1).

Elution was performed with 0.1 M phosphate buffer (pH 7.4) at 4°C, and radioactivities of every 2 ml fraction of eluate were determined.

Radioelectrophoresis was performed according to the agarose electrophoretic method of Deckert [4]. A mixture of 0.02 ml of serum, 0.01 ml of 125-I-insulin solution (total amount; ca. 160 μ U.) and 0.02 ml of veronal buffer (pH 8.6, 0.05 M) was incubated overnight at 4°C and a portion of the mixture was applied on agarose gel electrophoresis.

Radioimmuno-electrophoresis according to the meth-

Fig. 3. The Sephadex G-100 column chromatography of the mixture of the baby's (left side) or the mother's (right side) serum, radioinsulin and 0.02M phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin. The white column indicates the radioactivity and black column indicates protein content in 2 ml of fractions. The eluting solution is described in the right side of each figure

Experimental Methods

Titre of the insulin binding substance was determined with the Sephadex chromatographic method of Horiuchi *et al.* [3] and expressed in values of B/F. The mixture containing 0.2 ml of serum, 0.1 ml of 125-I-insulin solution (total amount; ca. 4 μ U.) and 0.7 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin was incubated at 4°C for 3 days and applied on a Sephadex G-100 column (1.0 $\times$ 15 cm).

od of Yagi and Maier [5] and specific immunoprecipitation were performed with antisera purchased from Beringwerke AG, Marburg, Lahn, Germany.

The autoradiography was performed by contact of slides with a medical X-ray film (Type Kx, Fuji Photo Industries Co., Tokyo, Japan) for 3 weeks.

Determination of serum free and total insulin levels was performed by radioimmunoassay after extraction by polyethylene glycol solution [6].

125-I-insulin with a high specific activity (150–300

mCi/mg) was obtained from Abbott Laboratories, Oak Ridge, Tenn. U.S.A. and purified by the method of Banerjee (7).

The blood sugar was determined by Hoffman's method on an Auto-Analyzer.

Experimental Results

Sephadex G 100 Column Chromatography

When eluted with 0.02 M phosphate buffer after the incubation and the application on the Sephadex column of mother's or baby's serum, as described in experi-

Radioelectrophoresis

With radioelectrophoresis of the mother's serum, all radioactivity was found in the gamma-globulin fraction. In the case of the baby's serum, most of the radioactivity was found in the gamma-globulin fraction and a small amount of the radioactivity in the alpha-globulin fraction (free insulin fraction). Also, in this test when a large amount of cold insulin (total amount: 100 mU.) was added to the incubation mixture, most of the radioactivity was found in the alpha-1-globulin fraction (Fig. 4).

These results indicate that the insulin-binding substance in their sera was contained in gamma-globulin.

Fig. 4. The protein and autoradiogram of the agarose gel electrophoresis of the baby's (left side) or mother's (right side) serum incubated with radioinsulin

mental method, most of the radioinsulin was eluted in the protein fractions and some part in the free insulin fractions. With 0.1 M glycine-HCl buffer (pH 3.0) as the eluent, most of the radioinsulin was eluted in the free insulin fractions. When a large amount of cold porcine insulin (total amount ca. 12 mU.) was added in the incubation mixture, most of the radioinsulin was eluted in the free insulin fractions (Fig. 3).

These results indicate that the binding substance in their sera was a high molecular substance which bound specifically to insulin and dissociated at a low pH.

Radioimmuno-electrophoresis

Radioimmuno-electrophoresis of the baby's and mother's sera against rabbit anti-whole human serum, anti-human IgG, anti-human IgM, anti-human IgA and anti-human IgD were performed. Precipitation lines were obtained except in IgD of the baby and the mother and IgA of the baby, but radioactivity of the precipitation line was detected only in IgG of mother and baby (Fig. 5).

This result indicates that the insulin-binding substance in their sera was contained in IgG.

Production of the Insulin-Binding Substance by Peripheral Lymphocytes in Vitro

The lymphocytes separated from 20 ml of the mother's peripheral blood were cultured in vitro at 38°C for 5 days, according to the method of Miyai *et al.* [8].

The supernatant of the culture was incubated with radioinsulin and the insulin-binding activity was determined with the same method as in the sera. The insulin-binding substance was detected in the supernatant of the culture of the mother's lymphocytes

and concentration with dialysis against 0.1 M phosphate buffer (pH 7.4) containing 20% gum arabic. The insulin-free serum was incubated with a constant amount of porcine radioinsulin (total amount; ca. 4 uU.) and various amount of cold insulins from human, porcine, bovine, bonito and tuna pancreas. After the incubation, the binding of the radioinsulin was determined with the Sephadex column chromatography.

Human and porcine insulins were bound more strongly than bovine insulin to the insulin-binding substance. Tuna and Bonito insulins cross-reacted less

Fig. 5. The protein stains and autoradiogram of the immunoelectrophoresis. The upper hole of each slide was filled with baby's (left side) or the mother's (right side) serum and the under hole was filled with control normal serum. The antiserum used is indicated in the right side of the figure

with or without phytohemagglutinin, but not in that of the control patient and the binding was dissociated with the addition of large amount of cold insulin (total amount: 12 mU.) (Table 2). The binding substance was also shown by agarose gel electrophoresis.

The Affinity of the Insulin-Binding Substance to Various Species of Insulin

The insulin-free sera of the mother and the baby were obtained by separation in Sephadex G-100 column chromatography (0.1 M glycine-HCl buffer, pH 3.0)

strongly than did any of human, porcine and bovine insulin (Table 3).

Heavy Chain of the Insulin-Binding Substance

As described above, by radioimmuno-electrophoresis, the binding substance was contained only in IgG. For further confirmation, radioimmunodiffusion using a larger amount of serum (10 μ l.) against anti-human IgG, anti-human IgM and anti-human IgA serum, with a similar technique to radioimmuno-electrophoresis, was performed. As shown in Table 4, in the baby's

and the mother's sera neither IgM nor IgA antibody was detected in contrast to that in sera from insulin-treated patients with comparable titres of antibodies. IgM antibody was detected in all sera and IgA antibody in some sera (Table 4).

Light Chain of the Insulin-Binding Substance

As shown in Fig. 5 in radioimmuno-electrophoresis, no radioactivity was detected in IgG-lambda-chain precipitate. For further confirmation, a specific precipitation test was performed. After the incubation of 0.2 ml of the mother's or baby's serum with 0.01 ml of

precipitate was higher as compared to the ratio of the lambda-chain precipitate and the ratio of the latter was equivalent to that of the control serum, the radioactivity of which was caused by non-specific co-precipitation of radioinsulin.

The result showed that the insulin-binding substance in the mother's and the baby's sera had pure IgG-Kappa-chain.

Dilution Test of Serum Extractable Insulin

The extracted insulin from the baby's or the mother's serum with the polyethylene glycol method was

Table 2. *Antibody production by lymphocytes*

Lymphocytes	Culture	Antibody titre (B/F)	
		without cold insulin	with cold insulin
Patient's mother	with PHA	0.35 ± 0.02	0.02
	without PHA	0.23 ± 0.03	0.01
Normal person	with PHA	0.02 ± 0.00	
	without PHA	0.02 ± 0.00	
No lymphocyte	with PHA	0.04	
	without PHA	0.03	

B/F: Bound insulin/Free Insulin

Cold insulin: 50 µg porcine insulin/ml added.

Lymphocyte culture: 4 ml of TC-199 solution with 20% calf serum, 0.1 ml of PHA-P solution, $1.0-1.5 \times 10^6$ of lymphocytes/ml, at 37°C for 5 days.

Table 3. *Species specificity of insulin-antibody reaction*

	Concentration of cold insulin added. (µU/ml)	B/F, Species of cold insulin added					
		None	Human	Porcine	Bovine	Tuna	Bonito
The mother's antibody	0	1.44					
	36.5		1.00	1.06	1.15		
	75		0.89	0.80	1.11		
	150		0.48	0.53	0.91		
	300		0.19	0.30	0.63	1.25	1.34
	600		0.18	0.16	0.35	0.86	1.02
The baby's antibody	0	0.92					
	37.5		0.67	0.65	0.65		
	75		0.52	0.46	0.61		
	150		0.24	0.23	0.46		
	300		0.01	0.08	0.29	0.74	0.76
	600		0.00	0.01	0.15	0.68	0.73

radioinsulin solution (total amount ca. 4 µU.) at 4°C for 3 days, rabbit anti-kappa-chain or anti-lambda-chain serum containing an approximately equivalent amount of antibody was added. After overnight incubation, the precipitate was washed thoroughly with a buffered saline solution, then the radioactivity and the protein in the precipitate was determined (Table 5).

In the serum from an insulin-treated patient, the ratio of radioactivity to the protein in the kappa-chain precipitation was substantially the same as that in the lambda-chain precipitate, but in the serum from the mother or the baby, the ratio in the kappa-chain pre-

precipitate was higher as compared to the ratio of the lambda-chain precipitate and the ratio of the latter was equivalent to that of the control serum, the radioactivity of which was caused by non-specific co-precipitation of radioinsulin.

The result showed that the insulin-binding substance in the mother's and the baby's sera had pure IgG-Kappa-chain.

Insulin Degradation Test

The extracted insulin from the mother's serum with the polyethylene glycol method was subjected to the degradation test of Elliott *et al.* [9, 10] with the insulin-specific peptidase from rat skeletal muscle.

The ratio of degradation was not different from that of the standard human insulin (Table 6).

Discussion

The insulin-binding protein in the sera of both baby and mother had the same characteristics on Sephadex chromatography, agarose gel electrophoresis and radio-immunoelectrophoresis as insulin-antibodies from insulin-treated diabetics and was produced by the mother's peripheral lymphocytes in vitro. These findings indicate that the substance is an insulin antibody although no previous insulin treatment had been administered to these patients.

Table 4. Radioimmunodiffusion

Serum	Antibody titre (B/F)	Anti-serum		
		IgG	IgM	IgA
Patient: baby	1.82	+++	—	—
Patient's mother	2.26	+++	—	—
Insulin-treated diabetics				
1.	2.62	+++	+	+
2.	3.90	+++	++	—
3.	1.75	+++	+	+
4.	1.08	+++	+	—
5.	1.25	+++	+	—
6.	1.44	+++	+	—
7.	1.60	+++	+	—
8.	1.48	+++	+	—

Antibody titre was determined with the method described in the text.

All radioimmunodiffusion was performed at the same time in the same conditions.

hypothesis that this insulin antibody was not produced against exogenous insulins, but produced probably against endogenous insulin.

The first evidence is that the insulin antibody has a simple immunoglobulin type (IgG-Kappa) which was not found in thirty cases of insulin-treated diabetics in our experience. Only one case of an insulin-treated diabetic with an insulin-antibody consisting of a single immunoglobulin type (IgG-Lambda) has been reported,

Fig. 6. Dilution test of baby's and the mother's serum insulins

Table 5. Specific precipitation of insulin antibody

Serum	Anti-K-chain serum			Anti-L-chain serum		
	Count	Protein	C/P	Count	Protein	C/P
Patient's mother	3092	32.0	96.61	126	21.2	5.94
Patient: baby	1080	22.2	48.67	78	17.0	4.59
Insulin-treated diabetic patient	4224	60.0	70.40	3149	44.0	71.56
Normal person	111	30.1	3.76	96	20.0	4.70

Count: Counts in the precipitate to 10^4 of total counts.

Protein: Protein in the precipitate in $\mu\text{g N}$.

C/P: Count/Protein.

Table 6. Degradation Test of Extracted Insulin

Insulin from	Insulin concentration ($\mu\text{U/ml}$)	Degradation rate (%)	
		30 min	60 min
Patients's mother	31.3	53.04 ± 0.08	56.87 ± 0.11
	61.0	47.54 ± 0.26	75.90 ± 0.04
Human standard	30.3	47.85 ± 0.08	58.18 ± 0.05
	54.4	61.83 ± 1.17	68.44 ± 0.04

Simple denial by the patient does not rule out previous insulin injections, as pointed out by Berkowitz *et al.* [11], but in this case, no reason for insulin therapy existed and the mother's abnormality of GTT was found after the baby's disease was recognized. In addition, two further pieces of evidence support the

by Faulk *et al.* [12], but the associated haemolytic anaemia, splenomegaly and hepatomegaly suggested that the patient had disturbances of the antibody producing mechanism.

The second evidence is that this antibody had a stronger affinity to human insulin and porcine insulin

than with bovine insulin. In this country, no pure porcine intermediately acting insulin preparation has been available and pure bovine or bovine-porcine intermediately acting insulin preparations have been used for treatment of diabetes. Insulin antibodies produced by these preparations had stronger affinities to bovine insulin without exception in our studies.

In the observed period, a gradual decrease of the baby's antibody titre ensued, but the mother's elevated antibody titre was retained. The clearance rate of the baby's antibody titre was approximately coincident to the clearance rate of mother's IgG from baby's serum after birth reported by Stiehm and Fudenberg [13] and not influenced by the administration of glucocorticoids. Thorell and Joergensen reported that the IgG insulin antibody transferred easily into foetal serum [14, 15]. These considerations, in addition to the fact that the baby's antibody had the same properties as the mother's antibody, make it highly probable that the baby's antibody was transferred transplacentally from the mother's serum.

There are two possibilities with regard to the mechanism by which this mother produced the insulin antibody against her endogenous insulin.

One possibility is the acquisition of antigenicity of her endogenous insulin by some kind of denaturation and cross-reaction of the antibody to her non-denatured endogenous insulin. This is supported by the experimental facts that homologous insulins are made antigenic by acid ethanol treatment and the induced antibodies cross-react with non-denatured, endogenous insulin [16, 17, 18]. The occurrence of such a minor denaturation might occur in the human body. In the dilution test and the degradation test, no abnormality of the mother's serum insulin was found, but a minor denaturation or minor part of denaturation which might be undetectable by such test, could cause the antibody production.

The other possibility is production of antibody by changing antibody-producing mechanisms. This seems to be supported by the fact that the antibody consisted of a monocomponent immunoglobulin, but against this, no other autoantibody could be detected in this patient. In addition, Nussenzeig *et al.* [19], Herd and Cebra [20], and Yamashita *et al.* [21] reported experimental production of antibodies with a single light chain against haptens after a long immunization. Therefore, the monocomponent antibody in this mother might be explained by immunization by her denatured endogenous insulin for a long period.

In this baby, no factor causing hypoglycaemia was found except for the presence of a large amount of insulin antibody and extractable insulin in her serum. Hirata *et al.* [22, 23, 24] and Folling and Norman [25] reported adult cases of hypoglycaemia with high titres of insulin antibody and large amounts of extractable insulin in their sera, but there was no previous history of insulin injection in their patients. In addition, Hirata *et al.* [23] reported the occurrence of hypo-

glycaemia in some animals immunized with insulin, even after the termination of insulin injection. He postulated that the hypoglycaemia was due to the presence of a large amount of inactive bound insulin and an occasional increase in active free insulin in serum by dissociation of the bound insulin. This explanation is applicable to this case.

The reason why the mother showed no hypoglycaemia despite a large amount of antibody could be explained in two ways.

The first hypothesis is that the mother's antibody is not completely saturated with endogenous insulin due to exhausted islet function produced by over-activity for a long period, so an increase in free insulin level hardly occurs. The second hypothesis is that adults are more resistant to hyperinsulinism due to more accomplished gluconeogenic mechanisms, an hypothesis invoked in the pathogenesis of idiopathic neonatal hypoglycaemia [26]. In this patient, however, no confirmatory data to support either hypothesis was obtained, so further investigations are required to clarify the mechanism of hypoglycaemia.

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