

# IMMUNOREACTIVE INSULIN AND INSULIN DEGRADING ENZYMES IN ERYTHROCYTES A Preliminary Report

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## SUMMARY

Immunoreactive insulin (IRI) and insulin degrading enzyme activity (IDEA) of the plasma and the corresponding erythrocyte lysate were estimated in 21 normal volunteers, 18 non insulin dependent diabetic patients (NIDDM), and 16 insulin dependent diabetics (IDDM). The erythrocytes contained several-fold higher concentrations of IRI than in plasma, both in normal and diabetic subjects. The values in controls ranged from 80 to 458 uU/ml against a range of 5 to 25 uU/ml in the corresponding plasma samples. The IRI contents of the diabetic patients were also similar. It showed no correlation to the fasting plasma glucose or the plasma IRI. Following an oral glucose load, no change occurred in the IRI content of the erythrocytes, unlike the changes seen in plasma. The IRI content of the lysate increased with dilution of the sample.

The IDEA was higher in diabetic patients compared to controls, especially so in the IDDM ( $P < 0.01$ ). It also showed more than one peak activity at different pH of the reaction buffer, indicating the possibility of a complex of enzymes.

Human erythrocytes contain large pools of IRI and its degrading enzymes. The significance of the pool of the insulin in non-target tissue needs to be studied.

**Key Words:** Immunoreactive insulin, erythrocytes, diabetes mellitus, insulin degrading enzymes.

## INTRODUCTION

Erythrocytes possess specific functional units such as insulin receptors<sup>1-4</sup> and insulin degrading enzymes<sup>5-7</sup> although they are not a target tissue for insulin action. The insulin-specific receptors on the erythrocytes are identical in structure and function to those on insulin-sensitive tissues such as fat cells.<sup>8-10</sup> The presence of these functions specifically related to insulin metabolism suggests that erythrocytes may contain insulin and may be involved in its metabolism. There have been a few reports (reviewed in Ref 11) on the insulin content of erythrocytes.

This paper reports the preliminary findings of a study aimed at identification and measurement of immunoreactive insulin (IRI) and the insulin degrading enzyme activity (IDEA) in the erythrocytes of normal controls and diabetic patients.

## MATERIAL AND METHODS

Subjects with normal glucose tolerance were studied as controls. The diagnosis of diabetes and classification of the diabetic patients into non-insulin dependent (NIDDM) and insulin dependent (IDDM) types were based on the WHO study group report criteria<sup>12</sup>. The study was done only in newly diagnosed NIDDM pa-

tients to avoid the effects of any therapeutic intervention.

**Sample preparation:** Blood samples were drawn after an overnight fast from normal control subjects ( $n = 21$ ; M:F 12:9, mean age  $34 \pm 10$  years), NIDDM patients ( $n = 18$ ; M:F 11:7, mean age  $43 \pm 8$  years) and IDDM patients ( $n = 16$ ; M:F 9:7, mean age  $32 \pm 12$  years) for the measurement of plasma glucose, HbA1c and insulin in plasma and erythrocytes. In 19 controls and 15 NIDDM patients, samples were also drawn at 1 hour and 2 hour intervals following ingestion of a 75 g glucose load.

Blood was collected in EDTA and aliquoted into different tubes for measuring glucose HbA1c and insulin. Two ml blood was collected in heparin for the measurement of insulin degrading enzyme activity (IDEA)<sup>5</sup>. Plasma glucose was estimated by glucose oxidase method (reagents from Boehringer Mannheim, West Germany). The measurements were made using the Modulab System (Boehringer Mannheim, West Germany). HbA1c was measured by the colorimetric procedure of Eross *et al*<sup>13</sup>. Haemoglobin content of the lysates were measured using Drabkin's reagent.<sup>14</sup>

**Samples for IRI measurement:** Measured aliquots of blood (3 ml) were used. The plasma was separated soon after collection of the blood by centrifugation at 3000 rpm for 10 minutes. The white cell layer was removed. Care was taken to avoid disturbing the packed red cells. The red cells were then washed 5 times with 10 ml of cold normal saline by careful mixing and centrifugation at 4°C. Each time, care was taken to avoid any

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loss of cells while removing the saline. Finally, the washed cells were lysed with cold distilled water making the total volume of the lysate equal to that of the original blood sample (3 ml). The lysed samples were centrifuged once again, at 3000 rpm for 15 min to remove any debris. The supernatant was stored at  $-20^{\circ}\text{C}$  till the assay was done.

Free insulin was estimated in the plasma samples of IDDM patients by extracting the samples with equal volume of 30% polyethylene glycol before measurement of IRI.<sup>15</sup>

**Samples for IDEA measurements:** Two ml of the blood sample collected in heparin was centrifuged, plasma was removed and approximately 1 ml of the erythrocyte suspension was washed thrice with cold normal saline. Finally, the cells were lysed with 10 volumes of cold distilled water. The lysate was centrifuged to remove the debris and 5 ml of the aliquot was frozen for the enzyme measurement. Haemoglobin (Hb) content of the lysate was measured on the same day. Use of EDTA was avoided in collecting the sample for the enzyme assay to prevent any inhibition of the activity by the compound.

**IRI estimation:** IRI was measured in the plasma and lysate using the method of Herbert *et al*<sup>16</sup>. Dextran coated charcoal was used to separate the free and bound forms. The intra and inter assay coefficients of variation for plasma IRI were 6.8% and 8.9% respectively and for the lysates, 7.6% and 9.6% respectively. The sensitivity of the assay was 2 uU/ml. Assays were carried out with 0.1 ml of the plasma and 0.1 ml of 1/10 diluted lysate. IRI measurements in the plasma and the corresponding lysate were carried out in the same batch of assay. The lysate was diluted 1:2, 1:5, 1:10, 1:20, and 1:40 with cold distilled water at the time of the assay to study the effects of dilution on the IRI of the lysate.

**IDEA estimation:** IDEA was estimated in the lysates by the trichloroacetic acid (TCA) precipitation method.<sup>5</sup> The incubation mixture (2 ml) contained tris/HCl buffer (10 mMol/l, pH 7.4), human albumin (1 mg/ml), highly purified pork insulin (Novo MC - Actrapid 100 mu/l), <sup>125</sup>I as tracer and 0.5 ml haemolysate.

The mixture was incubated at  $37^{\circ}\text{C}$  for 20 min; aliquots were removed at 0 and 20 min and equal amounts of 5% TCA were added to precipitate the proteins. The concentration of degraded insulin was calculated from the percentage of radioactivity in the TCA soluble fraction. The specific activity was calculated from the Hb concentration of the lysate. One unit of IDEA was defined as 1 uU insulin degraded/min per mg Hb. IDEA was calculated as per the original procedure in which the 200 uU of insulin added to the reaction mixture was taken for the calculation. The results are shown as IDEA-1. The activity was also calculated using the 200 uU plus the concentration of insulin (uU) present in the aliquot of lysate added. The values are shown as IDEA-2. To study the influence of different pH, IDEA was estimated in the erythrocyte lysates of 7 controls and 7 diabetic patients using Tris-HCl buffer (10 mmol/l) with different pH (7, 7.4, 8.5, 9, 10 and 10.5). (Fig-2).

**Statistical analysis:** Wilcoxon rank sum test was used for group comparisons. The difference between the group means was compared using one-way ANOVA. Pearson's correlation analysis was also used.

## RESULTS

Fasting plasma glucose, HbA1c, fasting plasma IRI and corresponding IRI in the erythrocyte lysates, and the IDEA of controls, NIDDM patients and IDDM patients are given in Table 1.

Table 1: Fasting plasma glucose, plasma IRI, erythrocyte IRI and IDEA in controls and diabetic patients

Parameters	Controls (n = 21)		NIDDM patients (n = 18)		IDDM patients (n = 16)	
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median
Plasma Glucose (mg dl)	96 $\pm$ 9	96.0	204 $\pm$ 80	174.5	205 $\pm$ 58	220.0
HbA1c (%)	7.1 $\pm$ 0.9	6.9	10.1 $\pm$ 1.6	10.2	10.8 $\pm$ 1.8	10.7
Plasma IRI (uU ml)	23 $\pm$ 9	23.0	21 $\pm$ 10	20.0	11 $\pm$ 7	12.0*
Lysate IRI (uU ml)	175 $\pm$ 104	150.0	180 $\pm$ 90	160.0	204 $\pm$ 122	150.0
IDEA-1	77 $\pm$ 25	74.0	87 $\pm$ 26	83.0	115 $\pm$ 40	109.0*
IDEA-2	112 $\pm$ 44	106.0	124 $\pm$ 32	121.0	175 $\pm$ 81	169.0*

\*Free IRI

#P < 0.001 compared to control and NIDDM patients

IDEA = Insulin degrading enzyme activity

IDEA-1 = Before correction for lysate IRI

IDEA-2 = After correction for lysate IRI

As a routine, IRI content of the lysates was estimated using 1/10 dilution as the dilution studies showed a linear increase in the values upto 1/10 dilution both in controls and diabetics (see below). The IRI content was also well within the sensitive range of the assay. The values reported in this paper are with 1/10 diluted lysate, unless otherwise specified.

The IRI content of the erythrocytes in controls was several-fold higher than the corresponding plasma concentration ( $175 \pm 104$  vs  $23 \pm 9$  uU/ml). Similar values were noted in the NIDDM patients ( $180 \pm 90$  vs  $21 \pm 10$  uU/ml). The plasma free insulin concentrations in the IDDM patients were lower compared to control and NIDDM patients ( $11.0 \pm 7$  uU/ml;  $P < 0.001$  vs both). The erythrocyte IRI showed no correlation either to the fasting plasma glucose ( $r = 0.29$  in controls and  $0.1$  in NIDDM patients) or to the fasting plasma IRI ( $r = 0.163$  in controls and  $0.06$  in NIDDM patients).

Table 2 shows the IRI values in erythrocytes in response to glucose, in controls and NIDDM patients. In contrast to plasma IRI, erythrocyte IRI showed no significant change with glucose stimulation.

With increasing dilution of the haemolysate the IRI values also increased, both in the controls and in diabetic patients (Fig. 1). The increase was linear upto a dilu-

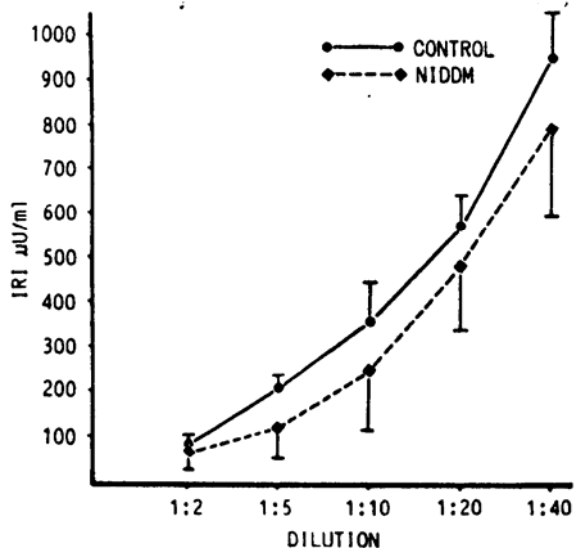


Fig. 1. Effect of dilution on the IRI levels of the hemolysate.

Table 2: Plasma glucose, plasma IRI and RBC IRI during glucose tolerance test in controls and NIDDM patients

	Plasma Glucose (mg/dl)			Plasma IRI (uU/ml)			RBC IRI (uU/ml)		
	Fasting	1 hour	2 hour	Fasting	1 hour	2 hour	Fasting	1 hour	2 hour
Controls (n = 19)	90 ± 23	131 ± 19	104 ± 20	22 ± 8	106 ± 72	67 ± 45	166 ± 110	178 ± 122	162 ± 93
NIDDM (n = 15)	198 ± 75	340 ± 78	335 ± 109	21 ± 10	84 ± 39	61 ± 33	180 ± 93	182 ± 87	198 ± 100

Values are mean ± SD

tion of 1/10. The pattern of increase in IRI activity with dilution was found to be similar both in controls and diabetic patients.

The IDEA was higher in the diabetic patients compared to the controls, especially so in the IDDM patients (Table 1). IDEA showed no correlation either to the fasting plasma glucose or the plasma IRI in the controls as well as in the NIDDM.

The effect of pH on the IDEA is shown in Fig 2. Maximum enzyme activity was seen at pH 7.4, both in controls and diabetic patients. Thereafter a gradual reduction was seen with increasing pH upto pH 10. The value increased again at pH 10.5. In the lysates from the patients also, the second peak was observed at pH 10.5.

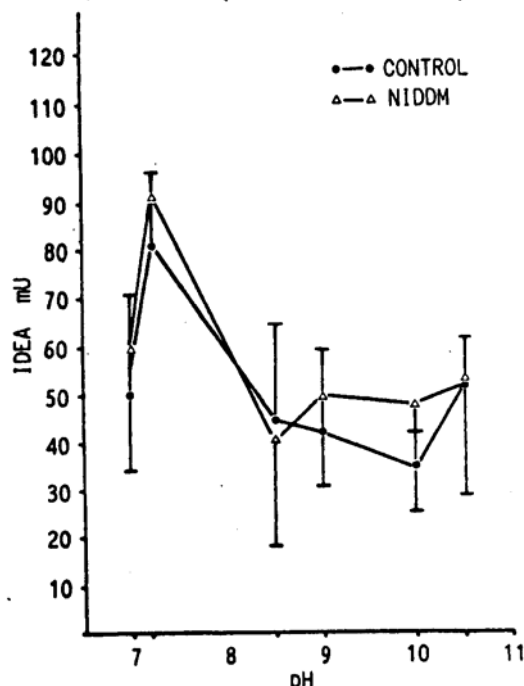


Fig. 2. Effect of pH on the IDEA of the hemolysate.

## DISCUSSION

This study shows that the erythrocytes of normal individuals contain a high concentration of immunoreactive insulin. The values in controls ranged from 80 to 458 uU/ml against a range of 5 to 25 uU/ml in the corresponding

plasma samples. These values agree well with those reported by Matulyavichyus *et al.*<sup>11</sup> The IRI content of the erythrocytes was similar in the diabetic individuals also. It showed no correlation either to the fasting plasma glucose or to the fasting plasma IRI. Following an oral glucose load, no change occurred in the content of IRI in erythrocytes, unlike the changes seen in the plasma. Thus the acute changes occurring in the circulating level of plasma insulin resulting from pancreatic secretion of the hormone appears to have no influence on the erythrocyte content of the hormone. This is in contrast to the observation of other workers who reported a decrease in IRI concentration after oral and intravenous glucose administration in healthy people, but an increase in individuals with impaired glucose tolerance and diabetes.<sup>11</sup> In this study, the IRI in the lysates of IDDM patients was higher than those in the control or NIDDM subjects. This finding differs from the earlier observation that it was significantly decreased in IDDM patients.<sup>11</sup> The reasons for the different observations are not clear.

The implication of the finding that the IRI activity increased with dilution of the lysate also needs to be studied further.

The fact that the erythrocytes contain large concentrations of IRI and IDEA is interesting. The insulin present in the human erythrocytes has been found to be identical to the native insulin in its molecular weight by gel filtration studies.<sup>11</sup> The physiological significance of the presence of high contents of both the substrate and the enzyme in the tissue is not known and warrants further studies. In the estimation of IRI *in vitro*, the IDEA does not interfere with the assay as the enzyme is inactive at the incubation temperature of 4°C.<sup>11</sup> We have also confirmed the observation that the enzymes are inactive at 4°C. As the lysates contain considerable amount of IRI also, its concentration needs to be accounted for in the calculation of the IDEA. The mean values corrected for this additional IRI content in the reaction mixture (IDEA-2) were thus higher than the values obtained using the concentration of 200 uU of insulin added to the mixture (IDEA-1).

No correlation was observed between the plasma insulin levels and the IDEA in controls and diabetic patients. Reports on this aspect are conflicting. Fratino *et al.*<sup>3</sup> observed no correlation between serum IRI and the IDEA in erythrocytes of controls, whereas Standl and Kolb<sup>5</sup> observed a significant positive correlation between these two parameters. In NIDDM patients these parameters were found to be inversely correlated by both groups of workers.

The presence of more than one activity (IDEA) peak at different pH indicates that the activity in the lysate

may be heterogenous. It may be a complex containing more than a single enzyme. This observation corroborates the finding of Matulyavichyus *et al.*<sup>11</sup>

The significance of large amounts of IRI and the IDEA in the erythrocytes is speculative at present. Such large concentrations of the hormone could only have accumulated over a period of time and whether this pool of the hormone has any metabolic implications merits further study.

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