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*J Clin Invest.* 1974;54(6):1323-1328. <https://doi.org/10.1172/JCI107878>.

**Research Article**

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# Decreased Insulin Binding to Lymphocytes from Diabetic Subjects

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**ABSTRACT** We have studied insulin binding to circulating lymphocytes isolated from 20 untreated adult, nonobese, nonketotic, diabetic subjects with fasting hyperglycemia, 20 normal subjects, and four patients with fasting hyperglycemia secondary to chronic pancreatitis. The results of these studies show that lymphocytes from diabetic patients have decreased ability to specifically bind insulin when compared to lymphocytes from normal subjects. For example, when lymphocytes from diabetic patients and a trace amount of [ $^{125}$ I]insulin ( $3.3 \times 10^{-11}$  M) were incubated, binding was less than 50% of the value obtained with lymphocytes from normal subjects ( $2 \pm 0.2\%$  vs.  $4.2 \pm 0.4\%$ ). Furthermore, the data show that lymphocytes from diabetic patients have only 1,200 insulin receptor sites per cell compared to 2,200 sites per cell for lymphocytes from normal subjects. Competitive inhibition studies using unlabeled insulin indicate that the affinity for insulin of lymphocytes from both groups is comparable. Consequently the decreased insulin binding of diabetics' lymphocytes is primarily due to a decreased number of available receptors rather than decreased binding affinity. This decreased insulin binding is not due to chronic hyperglycemia since insulin binding to lymphocytes, obtained from four patients with fasting hyperglycemia secondary to chronic pancreatitis, was completely normal. The possibility that some factor present in the plasma of diabetic patients could cause decreased insulin binding also seems unlikely since we could demonstrate no *in vitro* effects of diabetics' plasma on insulin binding. Lastly, the proportion of lymphocytes which were thymus derived and bone marrow derived were the same in each of the study

groups indicating that differences in lymphocyte subpopulations do not account for our results.

In conclusion: (a) lymphocytes from nonobese, untreated, adult diabetic patients with fasting hyperglycemia demonstrate a decreased ability to bind insulin; (b) this decreased insulin binding to lymphocytes obtained from diabetic patients can be accounted for primarily by an absolute decrease in the number of available receptor sites per cell; and (c) these data suggest that this defect in insulin binding is a primary phenomenon.

## INTRODUCTION

Previous studies have indicated that there is an attenuated fall in plasma glucose level when nonobese, nonketotic, adult diabetic patients with fasting hyperglycemia are given an intravenous injection of insulin (1). This suggests that these patients are resistant to the action of insulin. Since it has been shown that the initial step of insulin's action is binding to specific protein receptors located on the cell plasma membrane (2), it is possible that this decrease in insulin action might be due to decreased insulin binding. In this regard, insulin binding to receptors has been studied in man, and recent reports have indicated a defect in insulin binding to isolated circulating lymphocytes from obese insulin resistant subjects (3, 4). Consequently, in an attempt to see if decreased insulin binding is associated with the insulin resistance of diabetic patients we have studied insulin binding to lymphocytes from 20 normal adults, 20 nonobese, nonketotic, adult, diabetic subjects with fasting hyperglycemia, and four patients with hyperglycemia secondary to chronic pancreatitis.

## METHODS

*Materials.* Porcine monocomponent insulin was generously supplied by Dr. Ronald Chance of Eli Lilly and

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*Received for publication 10 April 1974 and in revised form 2 July 1974.*

TABLE I  
Clinical Characteristics of the Three Study Groups\*

	n	Sex		Age	Rel. wt.	Fasting plasma glucose	Fasting plasma insulin
		M	F				
Diabetics	20	15	5	50±2	1.01±0.04	203±18	21±2
Range				42-63	0.83-1.25	130-302	
Normals	20	11	9	45±2	0.97±0.04	89±6	11±1
Range				27-61	0.85-1.23	75-101	
Hyperglycemic pancreatitis	4	4	—	51±4	0.95±0.02	169±10	9±2
Range				40-60	0.90-1.01	125-260	

\* Mean ± SE.

Company (Indianapolis, Ind.). Na<sup>125</sup>I was purchased from New England Nuclear (Boston, Mass.), bovine serum albumin (fraction V) from Armour Pharmaceutical Company (Chicago, Ill.), and guinea pig anti-insulin antibody from Pentex Biochemical (Kankakee, Ill.).

**Subjects.** All patients were hospitalized on the Stanford General Clinical Research Center and fed isocaloric liquid formula diets (5) for at least 5 days before all studies. The study group consisted of 20 normal subjects, 20 patients with untreated, adult, nonketotic diabetes, and four patients who had diabetes secondary to chronic pancreatitis. The diabetic subjects were nonobese, and all of them had fasting plasma glucose concentrations > 130 mg/100 ml. The normal subjects were nonobese with normal oral glucose tolerance tests (6). Other than diabetes, no subject had any disease or was taking any agent known to affect carbohydrate metabolism. Table I summarizes some of the clinical characteristics of these subjects.

**Preparation of cells.** On the 6th day of hospitalization 120 ml of blood was drawn into heparinized glass tubes after an overnight fast. Plasma plus buffy coat were removed from the whole blood and transferred to a Ficoll-Hypaque gradient for cell fractionation according to the method of Boyum (7). Cells prepared in this manner contained less than 2% granulocytes and less than 4% monocytes. The remaining cells are lymphocytes, obtained in 80-90% yield with greater than 97% viability as determined by trypan blue exclusion. Cells are counted in triplicate by using a Neubauer chamber and diluted with buffer to the desired concentration for binding studies.

**Iodination of insulin.** [<sup>125</sup>I]insulin was prepared at specific activities of 150-250 μCi/μg by the method of Hunter and Greenwood (8) as modified by Freychet, Roth, and Neville (9). Approximate equimolar concentrations of Na<sup>125</sup>I and chloramine T were reacted, and the progress of the reaction was monitored by determining the amount of radioactivity absorbed to talc and precipitated by trichloroacetic acid. After completion of the reaction, the incubation mixture is fractionated on a cellulose column prepared in a Pasteur pipette (10). This method has been shown to yield a relatively pure preparation of biologically active monoiodinated [<sup>125</sup>I]insulin (9).

**Binding studies.** Lymphocytes (50 × 10<sup>6</sup> cells/ml) were incubated with insulin at 15°C in 0.5 ml of 25 mM Tris buffer (pH 7.6)-1% bovine serum albumin for 100 min. Bound and free insulin are separated at the end of the incubation period by the method of Rodbell (11). Duplicate 200-μl aliquots of the incubation mixture are layered over 150 μl of iced Tris buffer-2% bovine serum albumin in plastic

microtubes. Each tube is then centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 min, the supernates are discarded, and the radioactivity of the pellets is determined. Detailed characterization studies (12, 13), using lymphocytes from normal subjects, have demonstrated that the above incubation conditions give optimal steady-state binding, with little or no insulin or receptor degradation. Similar studies, using lymphocytes from diabetic subjects, show no differences in the time-course of the binding reaction or in the degree of insulin and receptor degradation. Details of the methods and procedures used in the characterization studies have been previously published (13).

**Calculations.** The mean number of receptor sites per cell was derived by submitting the data from the competitive inhibition studies to Scatchard analysis (14). By using bound radioactivity as a measure of the concentration of bound insulin (*B*), and the total insulin concentration minus *B* as the free insulin concentration (*F*), a plot of *B/F* to *B* (Scatchard plot) can be constructed. The *x*-intercept of the terminal slope of this plot gives the maximal amount of insulin bound. Receptor sites per cell is then calculated from the following formula:

$$\text{Sites per cell} = \frac{\text{moles insulin bound per ml}}{\text{cell concentration}} \times \frac{6.03 \times 10^{23} \text{ molecules}}{\text{mole}}$$

## RESULTS

Fig. 1 summarizes the ability of unlabeled insulin to competitively inhibit the binding of [<sup>125</sup>I]insulin to lymphocytes from 20 normal subjects (upper curve)

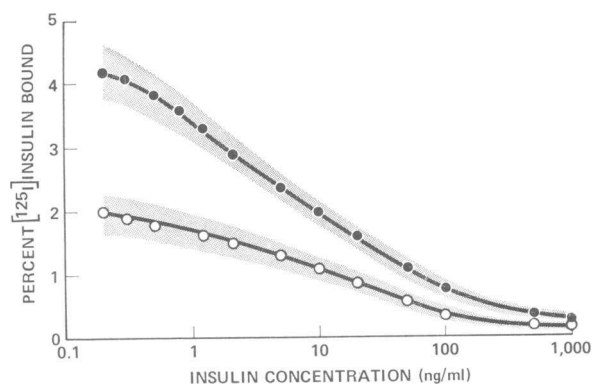


FIGURE 1 Comparison of [<sup>125</sup>I]insulin binding to lymphocytes obtained from 20 diabetic (○—○) and 20 normal (●—●) subjects. Cells (50 × 10<sup>6</sup> per ml) and [<sup>125</sup>I]-insulin (3.3 × 10<sup>-11</sup> M) are incubated at 15°C for 100 min in the absence of (initial point) and in the presence of increasing unlabeled insulin concentrations. Total insulin concentration is given on the horizontal axis and percent [<sup>125</sup>I]insulin specifically bound is on the vertical axis. Shaded areas represent ±1 SE. All data are corrected for nonspecific binding by subtracting the amount of [<sup>125</sup>I]insulin remaining in the cell pellets at 2 × 10<sup>6</sup> ng/ml insulin from the amount of [<sup>125</sup>I]insulin bound at all other insulin concentrations (4, 13, 21).

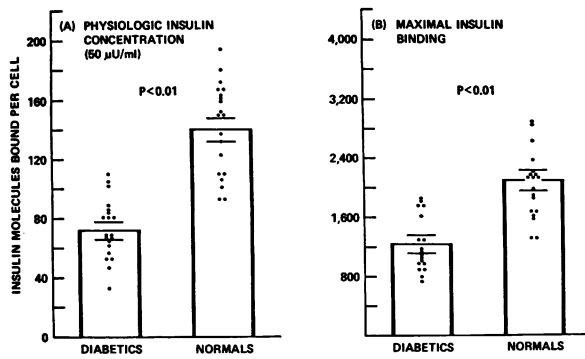


FIGURE 2 Comparison of the number of insulin molecules bound per cell at an insulin concentration of 50  $\mu\text{U/ml}$  (A) and the maximal number of molecules bound per cell (B) between lymphocytes from diabetic and normal subjects. Each point represents the data obtained from an individual experiment.

and 20 patients with idiopathic diabetes mellitus (lower curve). The upper curve demonstrates that  $4.2 \pm 0.4\%$ , 2.9–6.0% (mean  $\pm$  SE, range) of the [ $^{125}\text{I}$ ]insulin is bound to lymphocytes from normal subjects in the absence of unlabeled insulin. Furthermore, binding can be readily inhibited at physiologic insulin concentrations; i.e., [ $^{125}\text{I}$ ]insulin binding is 20% inhibited<sup>1</sup> at a total insulin concentration of  $1.2 \pm 0.1$  ng/ml ( $\sim 30$   $\mu\text{U/ml}$ ) and is 50% inhibited at  $9.0 \pm 1$  ng/ml ( $\sim 220$   $\mu\text{U/ml}$ ). These data represent specific insulin binding which is obtained by correcting the data for nonspecific binding (see legend to Fig. 1). In these studies, nonspecific binding averaged  $20.0 \pm 3\%$  (range 12–26%) of the total amount of [ $^{125}\text{I}$ ]insulin bound in the absence of unlabeled insulin. The lower curve summarizes the results obtained by using lymphocytes from diabetic subjects, and it can be seen that cells from diabetic subjects bind significantly less insulin ( $P < 0.01$ ) as compared to lymphocytes from normal subjects at every insulin concentration. Specifically, lymphocytes from diabetic subjects can bind only  $2.0 \pm 0.2\%$ , (range 1.2–3.1) of the [ $^{125}\text{I}$ ]insulin in the absence of unlabeled insulin. On the other hand, the ability of unlabeled insulin to inhibit [ $^{125}\text{I}$ ]insulin binding to lymphocytes from diabetics appears to be unchanged. For example, [ $^{125}\text{I}$ ]insulin binding is 20% inhibited<sup>1</sup> at a total insulin concentration of  $1.2 \pm 0.2$  ng/ml and is 50% inhibited at  $12.0 \pm 3$  ng/ml. Again, the data have been corrected for nonspecific binding which averaged  $23.0 \pm 3\%$  (range 11–30%) of the total amount of [ $^{125}\text{I}$ ]insulin bound in the absence of unlabeled insulin.

The data from Fig. 1 can be used to calculate the actual number of insulin molecules bound per cell at

<sup>1</sup>This degree of inhibition is statistically different from zero at the  $P < 0.01$  level by the paired  $t$  test.

any insulin concentration. This is done by converting the amount of insulin bound to molecules bound, and dividing by the cell concentration. Fig. 2 compares the maximal number of insulin molecules bound per cell, and the number of molecules bound at a physiologic insulin concentration (50  $\mu\text{U/ml}$ ), to lymphocytes from normal and diabetic subjects. At 50  $\mu\text{U/ml}$  (2A) lymphocytes from diabetic subjects bind about 50% as many insulin molecules per cell as do lymphocytes from normal subjects. Furthermore, since maximal binding occurs when all available insulin receptors are filled, the maximal number of insulin molecules bound per cell is equal to the number of receptors per cell. Therefore, since Fig. 2B demonstrates that lymphocytes from diabetic subjects can maximally bind only about 55% as many insulin molecules as lymphocytes from normals, one can conclude that lymphocytes from diabetics have 55% as many available insulin receptor sites per cell as do lymphocytes from normal subjects.

The ability of unlabeled insulin to inhibit the binding of [ $^{125}\text{I}$ ]insulin reflects the affinity of the binding reaction. Fig. 3 compares the percent inhibition in each individual study from both groups when 1, 10, 100, and 1,000 ng/ml of unlabeled insulin are added to the incubates. The similarity in the ability of unlabeled insulin to inhibit the [ $^{125}\text{I}$ ]insulin binding in both groups suggests that the binding affinity of lymphocytes from normal and diabetic subjects is also similar. Therefore, taken together, the data of Figs. 2 and 3 indicate that the decrease in insulin binding seen to lymphocytes from diabetic subjects is primarily due to a decreased

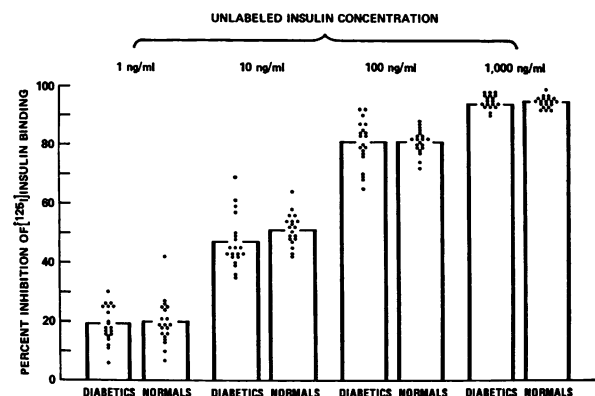


FIGURE 3 The percent inhibition of the [ $^{125}\text{I}$ ]insulin binding at unlabeled insulin concentrations of 1, 10, 100, and 1,000 ng/ml. All of the individual data points obtained from the 20 separate experiments performed in each group are plotted. At 1 ng/ml the degree of inhibition of [ $^{125}\text{I}$ ]insulin binding (mean inhibition: 20%) was significant ( $P < 0.01$ ) for both groups (paired  $t$  test). The data are derived from the inhibition experiments portrayed in Fig. 1 and are corrected for nonspecific binding.

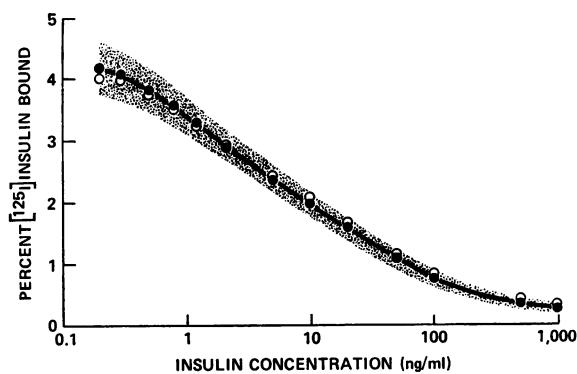


FIGURE 4 Comparison of [<sup>125</sup>I]insulin binding to lymphocytes from the 20 normal subjects (●—●) and four patients with hyperglycemia secondary to chronic pancreatitis (○—○). See legend to Fig. 1 for details of incubation.

number of available insulin receptors rather than to a decrease in the affinity of the receptor for insulin.

One could argue that this defect in insulin binding is not primary, but secondary to the hyperglycemic state. To answer this question we studied insulin binding to lymphocytes from four diabetic subjects with chronic pancreatitis. The hyperglycemia in these subjects is presumably secondary to pancreatic destruction. Fig. 4 compares the insulin binding ability of lymphocytes from these patients to that of lymphocytes from the normal subjects, and it is evident that insulin binding to cells from these subjects is normal. Thus, hyperglycemia per se does not appear to account for the decrease in insulin binding to lymphocytes from diabetic subjects as seen in Figs. 1 and 2.

To examine the possibility that some factor present in the plasma of these diabetic patients was responsible for the decreased insulin binding, we studied the *in vitro* effect of plasma from diabetic patients on insulin binding. In these studies cells were prepared from a normal subject and divided into three equal portions. Each portion was incubated for 3 h at 37°C, one with plasma from a normal subject, one with plasma from a diabetic, and the other with buffer. After the 3-h incubation period binding studies were performed in the usual manner. The results of these studies (five separate experiments) indicated that, compared to buffer, incubation of cells with plasma from either normal or diabetic subjects did not affect binding.

We have previously suggested that subpopulations of lymphocytes differ in their ability to bind insulin (13). To test the possibility that lymphocytes isolated from diabetics contain different proportions of thymus-derived (T)<sup>2</sup> versus bone marrow derived (B) cells

<sup>2</sup> Abbreviations used in this paper: B, bone marrow-derived; T, thymus-derived.

than lymphocytes isolated from normals, the T cell:B cell ratio was measured on cells isolated from each of the study groups. B cells were measured by specific immunofluorescent staining of surface immunoglobulins and T cells were measured by using a specific anti-T cell serum in a complement-dependent cytotoxicity assay by Dr. Arthur Bobrove and Dr. Samuel Strober, according to recently described methods (15). By this technique the normal percentage of total lymphocytes which are B lymphocytes is  $21.0 \pm 7$  (mean  $\pm$  SE). All of our subjects had normal values, and no difference in B cell:T cell ratios existed among our study groups.

## DISCUSSION

These results clearly demonstrate that lymphocytes isolated from adult, nonketotic, nonobese, diabetic patients with fasting hyperglycemia have decreased ability to bind insulin. For example, when lymphocytes and a trace amount of [<sup>125</sup>I]insulin were incubated, cells from diabetic subjects showed a highly significant greater than 50% decrease in insulin binding, and this decrease in binding was about the same at all insulin concentrations. Furthermore, these data also demonstrate that lymphocytes from diabetics bear only 55% as many receptors as do cells from normal subjects (1,200 sites per cell vs. 2,100 sites per cell). On the other hand, since unlabeled insulin inhibits [<sup>125</sup>I]insulin binding to the same degree to cells from either normals or diabetics, one can infer that the binding affinity of the lymphocyte insulin receptor is similar for both groups. Thus the decreased insulin binding of lymphocytes obtained from diabetic subjects seems primarily to be due to the absolute decrease in the number of available receptor sites per cell. Furthermore, this defect in insulin binding is not related to differences in subpopulations of lymphocytes since the B cell:T cell ratio was the same in all of our study groups.

Although these studies demonstrate decreased insulin binding to lymphocytes from diabetic patients they do not provide any information as to the relationship between decreased binding and insulin's biologic action. In this regard, however, Archer, Gorden, Gavin, Lesniak, and Roth (4) have recently demonstrated decreased insulin binding to lymphocytes isolated from obese human subjects, whose insulin resistance has been well documented (16). Furthermore, weight loss, which is known to decrease the degree of insulin resistance in obese individuals (5), is associated with an increase in insulin binding<sup>3</sup> (3). Thus, in man, decreased insulin binding to lymphocytes and insulin resistance seem closely related. With the above ob-

<sup>3</sup> J. A. Archer, P. Gorden, and J. Roth. Defect in insulin binding to receptors in obese men: amelioration with caloric restriction. Personal communication.

servations in mind, the data obtained in these current studies raise the possibility that there might also be an association between decreased insulin binding and decreased biologic action of insulin in these diabetic subjects.

These experiments were performed on lymphocytes isolated from patients who had been diabetic for varying periods of time, and, thus, it was not possible to determine if the decrease in insulin binding was a primary phenomenon or secondary to the hyperglycemic diabetic state. We attempted to answer this question in two ways. In the first place, we measured insulin binding to lymphocytes isolated from four subjects with chronic pancreatitis. These patients are also chronically hyperglycemic, but their hyperglycemia is thought to be secondary to pancreatic destruction. The data show that insulin binding to their lymphocytes was normal, indicating that chronic hyperglycemia does not result in decreased insulin binding. Secondly, lymphocytes from normal subjects were incubated with plasma from diabetic patients to see if some other factor in plasma was responsible for the decreased insulin binding. Within the experimental context defined, this series of studies indicates that preincubation of lymphocytes with plasma obtained from diabetic subjects does not decrease insulin binding. We believe that the data from these latter two studies suggest that decreased insulin binding to lymphocytes from diabetic patients is a primary phenomenon. On the other hand, the fasting plasma insulin levels of the diabetic subjects were elevated as compared to either the normal or pancreatic subjects, and Gavin, Roth, Neville, DeMeys, and Buell (17) have shown that prolonged elevations of in vitro insulin concentrations can lead to decreased numbers of insulin receptor sites on cultured lymphocytes. However, these patients were also certainly hypoinsulinemic in response to a glucose challenge (18, 19). Thus, it is difficult to know, at this point, what effect the ambient plasma insulin levels of our patients would have on their number of insulin receptor sites. Lastly, in our studies, in vivo binding of insulin to lymphocytes does not affect the subsequent measurement of insulin binding by occupying or "masking" receptor sites. This has been demonstrated by Archer et al. (4), who found that the addition of large amounts of insulin to whole blood, before isolating lymphocytes, did not affect insulin binding. The apparent explanation for this is that any insulin which might have been bound to the cell in vivo dissociates off the cell and is washed away during the lymphocyte isolation technique.

It must be emphasized that these studies were performed with isolated circulating lymphocytes, and that lymphocytes are certainly not an important target tissue

for insulin. Although animal studies have shown that the characteristics of insulin-lymphocyte binding (20) are similar to the characteristics of insulin binding to liver (21) and adipose tissue (22), the relationship between insulin binding to human lymphocytes and insulin binding to human insulin-responsive tissues remains to be established. Thus, before changes in insulin-lymphocyte binding can be accepted as a reflection of changes in insulin binding to human target tissues, comparative studies utilizing receptors isolated from such tissues will be necessary.

#### ACKNOWLEDGMENTS

We wish to thank Dr. Arthur Bobrove and Dr. Sam Strober for performing the lymphocyte typing studies. We also wish to thank Ms. Joyce Karst for her excellent technical work.

This work is supported in part by grants from the National Institutes of Health, HL 08506, and from the Veterans Administration.

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