Effects of Anti-insulin Receptor Autoantibodies on the Metabolism of Human Adipocytes

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SUMMARY

We studied the effect of sera from two patients who had an unusual form of diabetic syndrome with extreme insulin resistance on the metabolism of human adipocytes in vitro. The IgG fractions from sera A and B, which were obtained from two patients (1 and 2) with insulin-resistant diabetes, inhibited [^{125}I]insulin binding to human adipocytes and, at the same time, stimulated glucose oxidation and inhibited the lipolysis induced by levarterenol in human adipocytes. On the other hand, the IgG fraction from the C serum, which was obtained from patient 2 after her diabetic syndrome had completely disappeared as a result of immunosuppressive therapy, did not inhibit [^{125}I]insulin binding to human adipocytes, stimulate glucose oxidation, or inhibit lipolysis in human adipocytes. These facts suggest that these IgG fractions bind to or near the insulin receptor of human adipocytes, that they exhibit their insulin-like effect by binding to the insulin receptor in vitro, and, furthermore, that they are responsible for the extremely insulin-resistant diabetes. However, the apparent discrepancy between the effects of these IgG fractions on man in vitro and in vivo is puzzling and needs to be explained. DIABETES *27*:938-45, September, 1978.

There have been several reports of unusual forms of "diabetes" with insulin resistance.[1-4] Kahn et al. have reported six patients with an unusual syndrome of insulin resistance and acanthosis nigricans[5] and have shown evidence to explain this insulin resistance. That is, these investigators have shown the existence of circulating autoantibodies to the insulin receptor in the sera of patients with this syndrome and have suggested that these insulin-receptor antibodies cause impaired insulin binding, thus resulting in a clinical syndrome of extreme insulin resistance.

In addition, Kahn et al.[6] and we ourselves[7] have recently shown that the sera and IgG fractions from the patients with this unusual form of diabetic syndrome have a circulating antibody directed at or near the insulin receptor and that this antibody mimics the insulin effect in many aspects of the metabolism of rat adipocytes by binding to the insulin receptor in vitro. This apparent discrepancy between the effects of the antibodies on the insulin receptor in vivo and in vitro is puzzling. In this study, in order to establish whether or not this descrepancy is due to the differences in species, human or rat, we studied the effects of IgG fractions from two patients with this syndrome on [^{125}I]insulin binding, glucose oxidation, and lipolysis of human adipocytes. Furthermore, we obtained serum from one patient when her diabetic syndrome had disappeared after immunosuppressive therapy and studied the effect of this serum on the metabolism of human adipocytes.

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METHODS

Patients

The clinical courses of these two patients have been

reported by Kibata et al.[8] and Kawanishi et al.;[9] we will summarize them briefly here.

Patient 1 was a 44-year-old Japanese woman who suffered from extreme, insulin-resistant diabetes associated with acanthosis nigricans and a well-documented Sjögren's syndrome. She was neither obese nor lipoatrophic. Her blood glucose was not controlled in spite of the administration of up to 6,600 U. of regular insulin daily.

Patient 2 was a 45-year-old Japanese woman who also suffered from extreme, insulin-resistant diabetes associated with Sjögren's syndrome as well as some other immunologic abnormalities. Again, she was neither obese nor lipoatrophic. Her blood glucose was not controlled by the administration of 440 U. of Rapitard insulin, glibenclamide, or buformin. Immunosuppressive therapy with prednisolone and cyclophosphamide resulted in a decreased level of serum gamma globulin and a concomitant decrease in the blood glucose level. After immunosuppressive therapy for eight months, the diabetic syndrome disappeared completely. A circulating insulin-receptor antibody had been found in her serum, but it could not be detected after immunosuppressive therapy.

Materials

Porcine monocomponent insulin was purchased from Novo Industry, Copenhagen. [^{125}I]Na, [^{131}I]Na, and [1-^{14}C]glucose were purchased from New England Nuclear, Boston, Mass.; bovine serum albumin (fraction V) from Armour, Phoenix, Ariz.; human gamma globulin (fraction II) from Nutritional Biochemicals, Cleveland, Ohio; collagenase from Worthington Biochemicals, Freehold, N.J.; lactoperoxidase from Calbiochem; lactate dehydrogenase and glycerokinase from Boehringer-Mannheim, Germany; and levarterenol from Sigma Chemical, St. Louis, Mo.

Preparation of the IgG Fractions

Three kinds of sera were studied. Some biochemical data on these sera are shown in table 1. Serum A was obtained from patient 1, serum B was obtained from patient 2 at the beginning of immunosuppressive therapy with prednisolone and cyclophosphamide, and serum C was also obtained from patient 2 but nine months after the beginning of the immunosuppressive therapy. The immunoglobulins from each serum were precipitated by the use of 33 per cent saturated ammonium sulfate, reconstituted, and dialyzed against 0.005 M sodium phosphate buffer (pH 8.0). These preparations were then placed in a DEAE-cellulose (Whatman DE52) column (1 × 10 cm.) that had been equilibrated in a 0.005 M phosphate buffer (pH 8.0), and 1-ml. samples were collected. Most of the IgG molecules were eluted with the initial 10 ml. of this buffer. The remaining immunoglobulins were eluted with 0.1 M and 0.5 M NaCl, successively, in a 0.005 M phosphate buffer (pH 8.0). The highest protein concentration was obtained in fractions IV to VI: 4.1 mg. per milliliter in serum A, 2.6 mg. per milliliter in serum B, and 1.3 mg. per milliliter in serum C, as determined by the method of Lowry.[10] The immunoglobulins in the various fractions were characterized by the use of partigen (Japan Hoechst, Tokyo), and the first peak was found to be mainly IgG. The fraction with the highest protein concentration was used for all the following experiments as the IgG fraction from these sera.

TABLE 1

Some clinical data on the patients

	Immunoreactive insulin (μU./ml.)	Anti-insulin antibody (%)	Gamma globulin (gm./100 ml.)
Patient 1			
Serum A	980	N.D.*	2.7
Patient 2			
Serum B	—†	17.5‡	2.8
Serum-C	—	13.1	1.4

*The anti-insulin antibody was not detectable using both Sephadex column-gel filtration and polyethylene glycol methods.[8]

†Although immunoreactive insulin was not measured because of the presence of anti-insulin antibodies in the patient's serum, the level of C-peptide may indicate the hyperactivity of beta cells.[9] On admission, when no anti-insulin antibody was detected, the immunoreactive insulin in this patient was 340 μU. per milliliter.[9]

‡The anti-insulin antibodies were measured by the method of Welborn et al.[21] By this method, 3 to 6 per cent of the [^{125}I]insulin bound was considered to be negative, anti-insulin antibodies.[9]

Source of Tissue

Human subcutaneous adipose tissue was obtained during mastectomy. All patients fasted overnight. Anesthesia was induced with a short-acting barbiturate and maintained with halothane, nitrous oxide, and oxygen. The biopsies were usually obtained at an early stage of the operation. Immediately after excision, the biopsy specimens were placed in a vessel containing 20 ml. of a Krebs-Ringer bicarbonate buffer (KRB) with bovine serum albumin (BSA) (40 mg. per milliliter) and glucose (1 mM).

Preparation of Isolated Adipocytes

The isolated fat cells were prepared according to the method of Rodbell.[11] That is, the tissue pieces were placed in 10 ml. of a KRB buffer (calcium, 1.4 mM; pH 7.4) containing collagenase (3 mg. per milliliter), BSA (40 mg. per milliliter), and glucose (1 mM) and were then incubated in a water bath at 37° C. for 45 minutes. After the incubation, the isolated adipocytes

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were separated from the debris by filtration through a silk screen. The cells were washed three times in the same buffer. Adipocyte counts were performed using a microscope with a Bürker-Türk counting chamber.

Iodination of Insulin

The porcine insulin was iodinated by lactoperoxidase as has previously been described.[12] By this labeling method, 95 per cent of the radioactivity was precipitated by adding trichloroacetic acid to give a final concentration of 10 per cent and more than 85 per cent was precipitated by adding excess anti-insulin antibodies. The concentration of $[^{125}I]$iodoinsulin, which was used in binding studies, was radioimmunoassayed using $[^{131}I]$iodoinsulin by means of the double-antibody technique described previously.[12]

Incubation of Human Adipocytes with the IgG Fractions

As the maximum biologic effects of this IgG on rat adipocytes were demonstrated at the concentration of 0.06 mg. per milliliter in the previous study,[7] the concentrations of IgG fraction in the present study were fixed at 0.1 mg. per milliliter to find out whether these IgG fractions have any insulin-like effects or not. That is, this concentration of the immunoglobulin fractions were incubated with about 10 $\times$ 10^5 cells per milliliter of human adipocytes at 30° C. for 20 minutes. After a longer incubation time (60 to 90 minutes) or on incubation at 37° C., the inhibition of $[^{125}I]$insulin binding to human adipocyte increased by only 10 to 20 per cent; thus, we used the above conditions for all the experiments. After the incubation, fat cells were washed three times with the same KRB buffer. After washing, the binding studies, glucose oxidation study, and lipolytic study were performed as follows.

Binding studies. Isolated fat cells were suspended in KRB buffer (pH 7.4) containing BSA (40 mg. per milliliter) and then incubated with $[^{125}I]$iodoinsulin (0.5 ng. per milliliter) and unlabeled insulin in 3-ml. plastic test tubes in a shaking water bath at 24° C. Under the above conditions, optimal steady states for binding are achieved after 45 minutes of incubation with or without the preincubation of the immunoglobulin fractions. The incubations were terminated after 45 minutes by removing 200-μl. aliquots from the cell suspension and then rapidly centrifuging the cells in plastic microcentrifuge tubes that contained 100 μl. of dinonyl phthalate, as has been described by Gammeltoft and Gliemann.[13] After the centrifugation, the fat cells were packed in a layer over dinonyl phthalate oil interspersed between the cells and the lower aqueous layer. The tubes were cut through the oil layer and the radioactivity of the fat cells was determined. All the data were corrected for nonspecific binding by subtracting the amount of radioactivity remaining bound in the presence of 40 μg. per milliliter porcine insulin from the amount of radioactivity in the cell layer at all other insulin concentrations. The nonspecific binding in all the experiments was 10 to 15 per cent of the total binding and did not vary on incubation with immunoglobulin fractions.

Glucose oxidation studies. The ability of human adipocytes to oxidize glucose was measured essentially as has been described by Rodbell.[11] Human isolated adipocytes were incubated at 37° C. with $[1\text{-}^{14}C]$-D-glucose at a total glucose concentration of 1 mM in a KRB buffer (pH 7.4) containing BSA (40 mg. per milliliter). After two hours of incubation the generated $^{14}CO_2$ was trapped with hyamine hydroxide and counted in a liquid scintillation counter.

Studies of lipolysis. Isolated human adipocytes were incubated with a KRB buffer (pH 7.4) containing BSA (40 mg. per milliliter) and 1 mM glucose in polyethylene counting vials in a shaking water bath at 37° C. Lipolysis was induced by the addition of levarterenol (5 $\times$ 10^{-4} M), after which the incubation was continued for two hours according to the method of Jacobsson et al.[14] To stop the incubations by chilling, the incubation vials were transferred from the metabolic shaker to a rack placed in ice water. After the fat cells had been separated from the medium by centrifugation for 15 seconds in microcentrifuge tubes (capacity 1.5 ml.; Beckman), the medium was collected by needle-punching the tube bottoms. The amount of glycerol released into the incubation medium was taken as an index of the lipolysis. Medium glycerol was assayed by the enzymatic method of Garland and Randle.[15] The zero-time values were subtracted from the two-hour values to assess the net glycerol released during incubation.

RESULTS

Insulin-binding Studies

The IgG fractions from these insulin-resistant patients inhibited the $[^{125}I]$insulin binding to human adipocytes (figure 1), much as with IM-9 lymphocytes, 10^5 G pellets from human placenta, the circulating mononuclear leukocytes, and rat adipocytes.[7] However, the same concentration of the IgG fraction from serum C, which was obtained from patient 2 nine months after the beginning of immunosuppressive therapy when the diabetic syndrome had disappeared completely, had no inhibitory effect on the $[^{125}I]$insulin binding to human adipocytes (figure 1).

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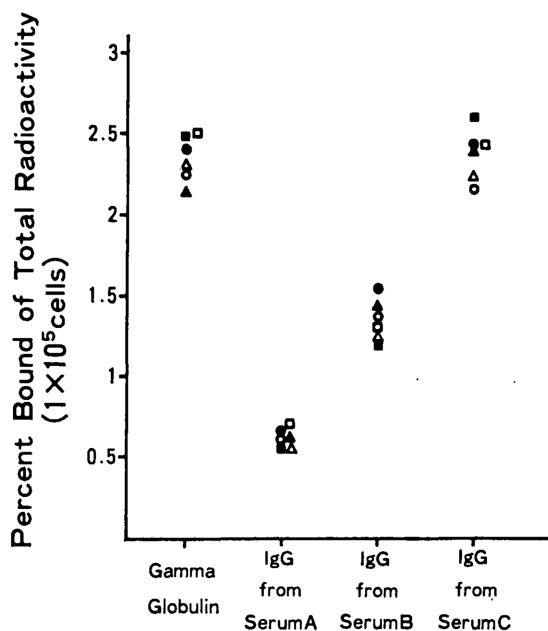

FIG. 1. Effects of the IgG fractions from the sera of patients with extreme, insulin-resistant diabetes on [125I]insulin binding to human adipocytes. Isolated human adipocytes (10 × 10^5 cells per milliliter) were preincubated with 0.1 mg. per milliliter of three kinds of IgG fractions from the patients and normal gamma globulin for 20 minutes at 30° C. Sera A and B were obtained from patients 1 and 2 when they both had extremely insulin-resistant diabetes. Serum C was obtained from patient 2 when her diabetic syndrome had disappeared completely after immunosuppressive therapy. After the incubation, the adipocytes were washed three times with a KRB buffer containing BSA (40 mg. per milliliter). After washing, [125I]insulin binding was studied for 45 minutes at 24° C.

Figure 2 shows the Scatchard plots[16] of the [125I]insulin binding to human adipocytes preincubated with the indicated IgG fractions. Although it is difficult to calculate the exact binding affinity from the curvilinear Scatchard plots because of the cooperativity phenomenon, the generally shallower slopes of the curves from the adipocytes preincubated with the IgG fraction from sera A and B and the coincidence of the horizontal intercepts of the terminal slopes of these four plots indicate that the decreased insulin binding to adipocytes preincubated with sera A and B are due to a decreased affinity of the receptor for insulin and that all four groups of cells have roughly the same number of the receptor sites per cell.

The Effects of IgG Fractions on Glucose Oxidation

Figure 3 shows the glucose oxidation by human adipocytes preincubated with the concentration of 0.1 mg. per milliliter of immunoglobulins in the absence and in the presence of the maximally effective concentration of insulin (100 ng. per milliliter).[17] As is shown in figure 3, the human adipocytes preincubated with IgG fractions from sera A and B oxidize glucose in the absence of insulin to the same extent that glucose is oxidized by adipocytes preincubated with normal gamma globulin in the presence of insulin at 100 ng. per milliliter. On the other hand, basal glucose oxidation of the human adipocytes preincubated with the IgG fraction from serum C is the same as that of the cells preincubated with normal gamma globulin, indicating the absence of insulin-like activity in the IgG fraction from serum C.

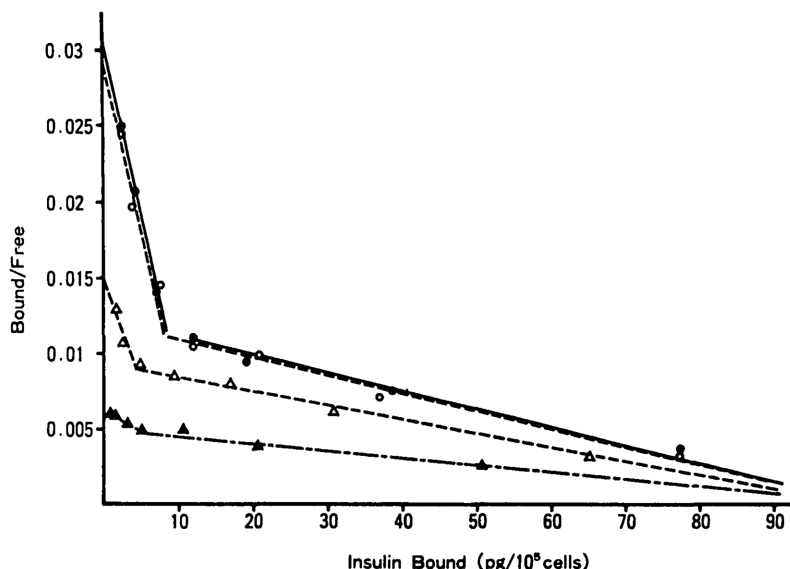

FIGURE 2

Scatchard plots[15] of the insulin-binding data for human adipocytes preincubated with 0.1 mg. per milliliter of normal human gamma globulin (closed circles) using the IgG fractions from sera A (closed triangles), B (open triangles), and C (open circles). The ratio of bound-to-free hormones is on the ordinate, while the bound hormone is on the abscissa.

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As shown in figure 3, studies of human adipocyte metabolism have been limited by a number of variables. For example, glucose utilization by human adipocytes is sensitive to the antecedent diet[17] and to the age of the donor.[18] Furthermore, it has been reported that cell rupture and the loss of the insulin responsiveness of human adipocytes during collagenase treatment occurs more easily than with rat adipocytes.[18] The observed variable metabolic rate during two-hour incubation may be due to a variable degree of such cell rupture. In addition, the type, the duration, and the depth of anesthesia may add further variability. Therefore, the data were analyzed as percentages of the insulin effect in the human adipocytes preincubated with normal gamma globulin. That is, first the basal value in the human adipocytes preincubated with normal gamma globulin was subtracted from that preincubated with the patient's IgG fractions in the presence or absence of insulin, and subsequently this difference was divided by the increment in the human adipocytes preincubated with normal gamma globulin by adding insulin (100 ng. per milliliter) (figure 4). With respect to the stimulation of the glucose oxidation, figure 4 clearly shows that the IgG fractions from sera A and B had almost the same

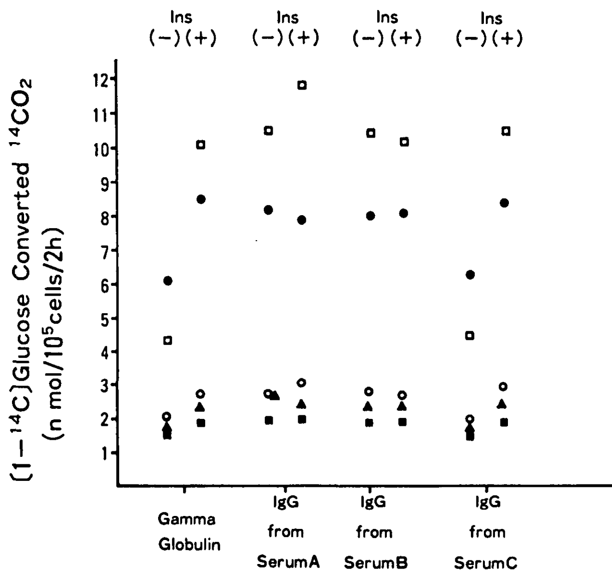

FIG. 3. Effects of the IgG fractions from the sera of patients with extreme, insulin-resistant diabetes on the glucose oxidation of human adipocytes. Isolated human adipocytes were preincubated with 0.1 mg. per milliliter of the IgG fractions as is described in the legend to figure 1. After washing, cells were incubated with 1 mM glucose in the absence and presence of 100 ng. per milliliter insulin for two hours at 37° C.

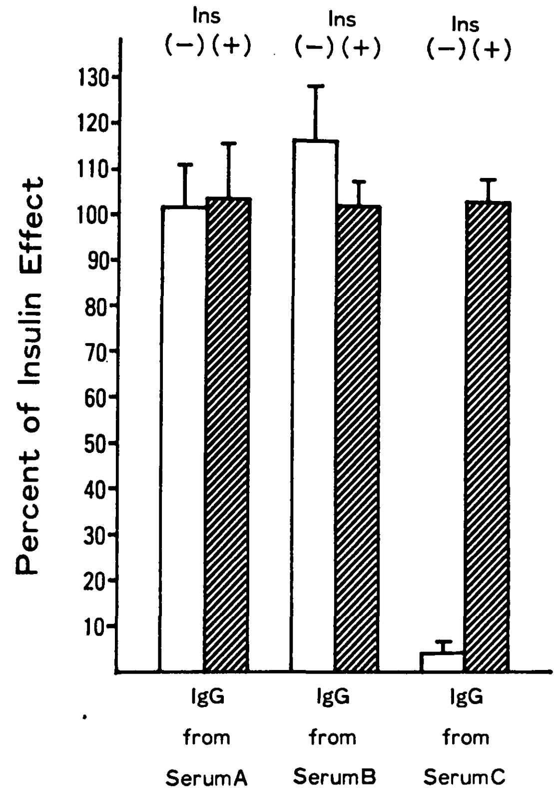

FIG. 4. Effects of the IgG fractions from the sera of the patients on glucose oxidation of human adipocytes. The data pictured in figure 3 were analyzed as percentages of the insulin effect (100 ng. per milliliter) on human adipocytes preincubated with normal gamma globulin. The values presented are the means ± standard errors of five separate experiments.

effect as insulin at 100 ng. per milliliter, which is the maximally effective dose in this human adipocyte study, and furthermore, that the effects of the IgG fractions and insulin (100 ng. per milliliter) on the stimulation of glucose oxidation in the human adipocytes are not additive. Also, as is shown in figure 4, the IgG fractions from serum C, which was obtained from patient 2 after the diabetic syndrome had disappeared completely after immunosuppressive therapy, had no effect on the glucose oxidation in human adipocytes.

Effects of IgG Fractions on Lipolysis

Figure 5 shows the lipolysis by human adipocytes preincubated with the concentration of 0.1 mg. per milliliter of immunoglobulins in the absence and the presence of the maximally effective insulin concentration (100 ng. per milliliter).[19] The lipolysis in the human adipocytes preincubated with the IgG fractions from sera A and B was more inhibited than that of the human adipocytes preincubated with normal gamma globulin and the IgG fraction from serum C.

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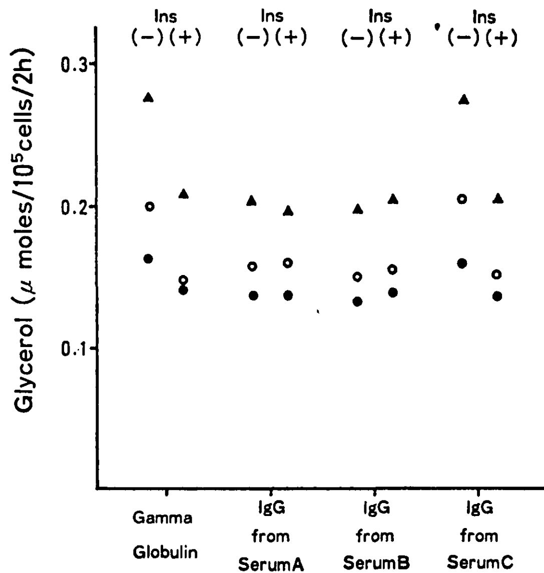

FIG. 5 Effects of the IgG fractions from the sera of patients with extremely insulin-resistant diabetes on lipolysis of human adipocytes. Isolated human adipocytes were preincubated with 0.1 mg. per milliliter of the IgG fractions as is described in the legend to figure 1. After washing, the cells were incubated with levarterenol (5×10^{-4} M) in the absence or presence of insulin (100 ng. per milliliter) for two hours at 37° C. The amount of glycerol released into the incubation medium was taken as an index of the lipolysis.

Insulin (100 ng. per milliliter) also inhibited the lipolysis of human adipocytes preincubated with normal gamma globulin and the IgG fraction from serum C. Furthermore, the inhibition of lipolysis in the human adipocytes preincubated with the IgG fractions from sera A and B was the same as that by insulin (100 ng. per milliliter) in the human adipocytes preincubated with normal gamma globulin and the IgG fraction from serum C. For the same reason as has been presented above for the glucose oxidation studies, the data were analyzed as percentages of the insulin effect in the human adipocytes preincubated with normal gamma globulin (figure 6). Figure 6 clearly shows that the IgG fractions from sera A and B had almost the same insulin effect (100 ng. per milliliter) with respect to the inhibition of lipolysis in the human adipocytes and that the IgG fraction from serum C, which was obtained when the diabetic syndrome had disappeared completely, had no effect on the lipolysis in human adipocytes.

DISCUSSION

In this study, the IgG fractions from patients who had extremely insulin-resistant diabetes accompanied by Sjögren's syndrome were found to have an insulin-like activity in the metabolism of human adipocytes. Furthermore, the IgG fraction from the serum obtained from a patient whose diabetic syndrome had disappeared completely after immunosuppressive therapy did not have the insulin-like activity in the metabolism of human adipocytes.

As shown in table 1, there were high concentrations of immunoreactive insulin in the sera of these patients. There is a possibility that this contamination of the immunoreactive insulin accounts for its insulin-like activity. However, the IgG fraction from serum A, in which we could detect no immunoreactive insulin, had insulin-like activities on the metabolism of human adipocytes. Although it is difficult to measure immunoreactive insulin in the IgG fractions from sera B and C accurately because of the presence of anti-insulin antibodies, it is likely that the contamination of insulin in these fractions is negligible since these IgG fractions were prepared in the same manner as the IgG fraction from serum A. As has been mentioned

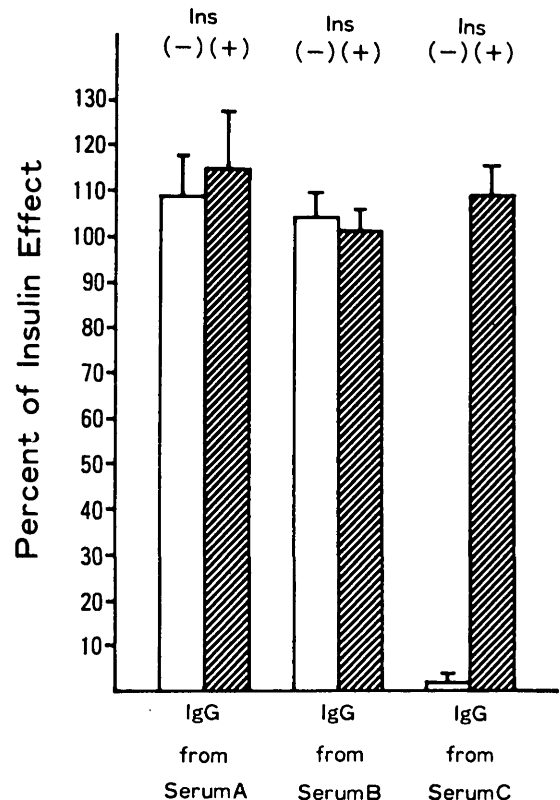

FIG. 6. Effects of the IgG fractions from the sera of the patients on lipolysis of human adipocytes. The data pictured in figure 5 were analyzed as percentages of the insulin effect (100 ng. per milliliter) on the human adipocytes preincubated with normal gamma globulin. The values presented are the means ± standard errors of three separate experiments.

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above, it is unlikely that the contamination of immunoreactive insulin accounts for its insulin-like activity.

In the study of the first patient with insulin resistance and acanthosis nigricans, Field et al. showed a marked increase of insulin-like activity in the serum of the patient.[1] It has been postulated that some of the insulin-like activity in this case was due to insulin bound to anti-insulin antibodies.[6] However, it is unlikely, in our cases, that the insulin-like activities in the sera of these patients were due to insulin bound to anti-insulin antibodies, for the anti-insulin antibody could not be detected in the serum of patient 1. Furthermore, in patient 2, the IgG fraction from serum C did not have any insulin-like activity in the metabolism of human adipocytes, although sera B and C had almost the same concentration of anti-insulin antibodies.

The IgG fractions from sera A and B, which were obtained from patients 1 and 2 when they had extremely insulin-resistant diabetes, inhibited the $[^{125}I]$insulin binding to human adipocytes and, at the same time, had insulin-like activity on the metabolism of human adipocytes. On the other hand, the IgG fraction from serum C, which was obtained from patient 2 when her diabetic syndrome had disappeared completely after immunosuppressive therapy, did not inhibit the $[^{125}I]$insulin binding to the human adipocytes nor did it have an insulin-like activity on the metabolism of the human adipocytes. These facts strongly suggest that the IgG fractions from the patients who have extreme, insulin-resistant diabetes bind to or near the insulin receptor of human adipocytes, that they exhibit their insulin-like effect by binding to the insulin receptor in vitro, and, furthermore, that they are responsible for the extreme, insulin-resistant diabetes in vivo.

As has been reported previously with regard to the rat adipocytes,[6,7] there is also an apparent discrepancy between the effects of these IgG fractions on the human adipocytes in vitro and in vivo; this discrepancy is puzzling. Originally, we raised the possibility that this discrepancy may be due to the different effect of this antibody between human and rat adipocytes.[7] Judging from the results of this study, it is unlikely that this discrepancy is due to any differences in effect by species, i.e. man or rat. It has been suggested that these antibodies have an insulin-like effect not only on the metabolism of adipocytes but also on liver and muscle in vitro.[6] Bar et al. reported that one of the patients with this syndrome died with intractable hypoglycemia, and they suggested that the insulin-receptor antibody may have had an insulin-like effect in vivo in her terminal stage.[20] This observation suggests that the insulin-like effect of an antibody on the insulin receptor might have been prevented in vivo by some unknown mechanism during her hyperglycemic stage. Although the exact mechanisms for this discrepancy between in vivo and in vitro are interesting, they remain to be explained.

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