Studies on Humoral Insulin Antagonists in Diabetic Acidosis

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In a previous paper an antagonist to insulin was described in the sera of patients in diabetic acidosis.[1] This factor was thought to be independent of the adrenal cortical hyperactivity and the lowered serum *p*H usually accompanying diabetic coma. Groen et al.[2] had reported previously that sera obtained from patients in diabetic acidosis were devoid of any insulin activity, but insulin added to such sera exerted its anticipated effect on the glucose utilization by the rat diaphragm. Consequently, these investigators concluded that the absence of an insulin effect in serum during diabetic acidosis was not attributable to an insulin antagonist. More recently Vallance-Owen et al.[3] have found that insulin added to plasma from diabetic patients requiring insulin and having a high blood sugar, but not in ketosis, did not increase the glucose utilization of the rat diaphragm. However, in these same patients when the blood sugar was normal, insulin added to the plasma exerted its usual effect. These authors suggest that many diabetics require insulin to overcome an insulin inhibitor in their plasma.

The present report concerns further studies which have been done to characterize the insulin antagonist found during diabetic acidosis.

METHODS

The method for determining the insulin effect was the same as was used previously[1] and is based on the ability of insulin to augment the glycogen deposition by the rat hemidiaphragm. Briefly, one hemidiaphragm was exposed to 1 ml. of serum and 1 ml. of 0.04 M phosphate-saline buffer (*p*H 6.8) containing 0.2 unit insulin for one minute while the control hemidiaphragm was exposed to only serum and buffer. Each hemidiaphragm was then washed twice in buffer solution and incubated for ninety minutes in a solution containing 0.4 per cent glucose in the same buffer. At the end of the incubation the glycogen content of each hemidiaphragm was determined. The increase in glycogen content in the rat hemidiaphragm exposed to insulin, expressed as *u*mole of glucose per gm. of diaphragm, is referred to as the insulin effect.

Sera used in the experiments were obtained from patients in diabetic acidosis at the time of their admission to the hospital before they had received any insulin therapy. In some cases a later specimen was obtained several hours after treatment for acidosis had been instituted. Serum was kept frozen at —4°C for many months without any loss of its ability to antagonize insulin.

Electrophoretic separation of the serum into its various protein components was done by continuous paper electrophoresis in a temperature-control apparatus at 8° C for thirty-six hours according to the method of Saroff.[4] The buffer system was sodium barbiturate of ionic strength 0.08 and *p*H 8.6. The current was 46 milliamperes and a double thickness of 12-inch sheet of Whatman #3 paper was used. The identity of the protein component in each fraction collected was determined by independent paper strip electrophoresis in the barbiturate buffer at room temperature for twenty-two hours using a current of 10 milliamperes and Whatman #1 paper. After the electrophoresis the strips were dried at room temperature and stained with bromphenol blue.

Lipoproteins were obtained by centrifugation of serum in the preparative ultracentrifuge at 15° C and 114,400 x g for eighteen hours. Prior to centrifugation, the density of the serum was adjusted to 1.21 by the addition of concentrated sodium chloride. Both the separated lipoproteins and the remainder of the serum were dialyzed for four hours against 0.04 M phosphate-saline buffer to reduce the salt concentration before they were each assayed in the rat hemidiaphragm system.

I^{131} labeled insulin was obtained from Abbott Laboratories and was dialyzed overnight against 0.04 M phos-

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phate-saline buffer at 14° C. At the end of dialysis 99 per cent of the radioactivity was trichloroacetic acid precipitable. The binding of insulin to the rat hemidiaphragm was measured as follows: After being weighed, one hemidiaphragm was exposed to 0.2 ml. of normal serum and 2 ml. of 0.04 M phosphate-saline buffer containing 0.2 units of nonlabeled insulin and 0.01 units of insulin-I^{131} with a total of 100,000 counts per minute. The other hemidiaphragm was exposed to a similar mixture except that 0.2 ml. of insulin antagonist serum was substituted for the normal serum. This amount of serum was capable of antagonizing the effect of 0.2 unit of insulin.[1] After one minute exposure each hemidiaphragm was washed twice in 30 ml. of phosphate-saline buffer for thirty minutes and then counted in a scintillation counter. The amount of insulin bound is expressed as counts per gram of tissue. Using this procedure, Stadie et al. have shown that insulin is rapidly and firmly bound to the hemidiaphragm and cannot be washed out.[5]

The presence of insulinase activity in the insulin antagonist serum was tested for by the procedure of Vaughan:[6] 0.2 ml. of normal serum and 0.2 ml. of antagonist serum were each incubated for sixty minutes at 37° C in 2 ml. of 0.1 M phosphate buffer (*p*H 7.4) containing 10 units of nonlabeled insulin and 0.001 units of insulin-I^{131}. At the end of the incubation the proteins were precipitated with an equal volume of 20 per cent trichloroacetic acid and the radioactivity in the supernatant solution counted in a scintillation counter. The total amount of radioactivity present at the beginning of the incubation was also ascertained. The insulinase activity is reported as the per cent of I^{131} initially present which became soluble in trichloroacetic acid. Five tenths ml. of rat liver homogenate in distilled water was used as a control since it is known to be a good source of insulinase.[6]

RESULTS AND DISCUSSION

Chemical Nature of the Inhibitor. Insulin antagonist activity was not lost as a result of overnight dialysis against sodium barbiturate buffer (*p*H 8.6) of ionic strength 0.1 at 18° C. The dialyzed serum was then separated into its protein components by paper electrophoresis. Serum obtained from Case VII was used for this study. It had previously been shown that 0.05 ml. of this serum inhibited the effect of 0.2 units of insulin and as little as 0.01 ml. had some activity.[1] An amount of albumin equivalent to 0.1 ml. of serum did not significantly lower the insulin effect (table 1). However, when alpha-globulins were tested the insulin effect was abolished by the equivalent of 0.02 ml. of serum. There was even some suggestive effect when one-tenth of this amount was used. Beta-globulins equal to 0.1 ml. of serum were devoid of demonstrable insulin antagonist activity. Only when twice this amount was used was there evidence of insulin antagonism. The small amount of activity present in this fraction can probably be explained by the incomplete electrophoretic separation of the protein fractions. The gamma-globulin fraction was also devoid of insulin antagonist activity. This insulin antagonist is either a protein or a smaller molecule somehow bound to a protein with an electrophoretic mobility similar to the alpha-globulins. Since the activity was in the alpha-globulins rather than the gamma-globulin fraction, it is apparent that it is a different material from the antibody which has been previously reported in the sera of some patients whose insulin resistance was not associated with acidosis or infection.[7, 8, 9, 10]

TABLE 1

Activity of various protein fractions of insulin antagonist serum

Fraction	Equivalent amount of whole serum ml.	Insulin effect micromoles (glucose equiv.) per gm. of tissue Mean±S.E.M.
Albumin	0.1	5.15±0.94 (6)*
alpha-Globulins	0.02	1.12±0.94 (9)
alpha-Globulins	0.002	3.70±1.49 (6)
beta-Globulins	0.2	2.52±0.97 (3)
beta-Globulins	0.1	4.13±2.68 (3)
gamma-Globulins	0.1	6.70±1.26 (8)
Normal Serum+	1	5.35±0.48 (23)

( )* Number of determinations
+ See bibliography reference 1.

Although the alpha-globulins are known to be rich in lipoproteins, the insulin antagonist is not a lipoprotein. In the presence of this fraction obtained from insulin resistant serum, the insulin effect was 4.66 micromoles/gm. of tissue as compared to —1.25 micromoles/gm. of tissue when the remainder of the serum was used (table 2). Repeated freezing and thawing did not decrease the activity of this insulin antagonist. Table 3 indicates that the antagonist was inactivated by heating at 100° C for four minutes, but not at 60° C for fifteen minutes.

Endocrine Relationships. Since insulin refractory diabetes has been produced by the administration of growth hormone to dogs,[11] and the diabetes which occurs in association with acromegaly in humans is often insulin resistant,[12] it was thought pertinent to test sera obtained from acromegalic diabetics for insulin antagonist activity. Although these two patients showed no fall in their blood sugar levels following an intravenous injection of

TABLE 2

Absence of insulin antagonist activity in lipoprotein fraction of serum from Case VII

Fraction	Equivalent amount of serum	Insulin effect micromoles glucose equiv./gm. of tissue Mean±S.E.M.
Lipoprotein	0.2 ml.	4.66±2.60 (3)*
Remaining proteins	0.2 ml.	−1.25±1.47 (6)

( )* Number of determinations

TABLE 3

Effect of heating on the activity of insulin antagonist serum

Treatment of serum	Equivalent amount of whole serum ml.	Insulin effect micromoles (glucose equiv.) per gm. of tissue Mean±S.E.M.
Untreated	0.1	−1.03±1.17 (6)*
Heated 15 min. at 60° C	0.5	0.65±0.14 (3)
Heated 4 min. at 100° C	0.1	4.53±0.95 (3)

( )* Number of determinations

0.1 unit of regular insulin/kg. of body weight, their sera did not contain any demonstrable insulin antagonist (table 4). Since it has been reported that adrenocorticotropin (ACTH) exerts a synergistic effect on the action of growth hormone,[13] one of the acromegalic patients (A.L.) was given a forty-eight-hour intravenous infusion of 200 units of ACTH. At the end of this infusion the serum still did not exhibit any insulin antagonist activity. It thus seems unlikely that the antagonist is related to growth hormone. However, until growth hormone in serum can be assayed directly, it is not possible completely to exclude it as the cause of the increased tolerance to insulin in diabetic acidosis. Previously, we have shown that this insulin antagonist is also independent of adrenal cortical hyperactivity.[1]

Other Relationships. In two cases of diabetic acidosis it was possible to obtain serum six and nine hours, respectively, after the institution of insulin therapy. Table 5 indicates that by this time it was no longer possible to detect any insulin antagonist. Either the antagonist had disappeared from the serum at these times or it had been effectively neutralized by the insulin which had been administered as therapy for the acidosis. On the basis of information available, we are unable to decide between these two hypotheses.

Marsh and Haugaard have previously reported an insulin antagonist in the sera of diabetic patients whose insulin resistance was not associated with either acidosis

TABLE 4

Inability of serum from diabetic acromegalics to inhibit insulin effect

Source of serum	Amount of serum ml.	Insulin effect micromoles (glucose equiv.) per gm. of tissue Mean±S.E.M.
H.R. 52 yr. female	1	5.93±0.95 (6)*
A.L. 55 yr. female	1	5.24±0.64 (6)
A.L.—at end of 48 hr. infusion of 200 units ACTH	1	5.41±1.27 (6)

( )* Number of determinations

TABLE 5

Time relationship between disappearance of insulin antagonism and onset of therapy for diabetic acidosis

Source of serum	Amount of serum ml.	Insulin effect micromoles (glucose equiv.) per gm. of tissue Mean±S.E.M.
Case VIII		
9-13-55 10:00 a.m.	1	1.74±1.82 (3)*
9-13-55 7:00 p.m.	1	5.69±0.13 (3)
Case IX		
2-21-56 4:00 p.m.	1	1.40±0.43 (8)
2-21-56 10:00 p.m.	1	4.40±2.56 (3)
Normal serum	1	5.35±0.48 (23)

( )* Number of determinations

or infection.[14] They found that if the hemidiaphragm was exposed to insulin resistant serum and then was subsequently immersed in an insulin solution, there was no inhibition of the insulin effect. This indicated that the factor which they were studying in serum had to be present at the same time the diaphragm was exposed to insulin in order to exert its antagonism. In contrast to their observation, table 6 shows that the humoral factor present during diabetic acidosis was capable of inhibiting the effect of insulin whether diaphragm was exposed first to either insulin or serum. These findings indicate that the insulin antagonist in the serum during diabetic acidosis was bound rapidly and firmly by the diaphragm and then exerted its anti-insulin effect. Even if the insulin was bound to the diaphragm first, the antagonist was still capable of inhibiting its action. These experiments also show that the antagonist did not compete with insulin for binding sites on the diaphragm, nor did it exert its effect by preventing the diaphragm from binding insulin. Further support for this latter conclusion was obtained by measuring the amount of insulin-I^{131} bound to the rat diaphragm in the presence of normal and antagonist serum. Table 7 demonstrates that the same amount of insulin was bound by the diaphragm whether insulin

TABLE 6

Ability of insulin antagonist serum to abolish insulin effect when diaphragm is exposed to it and insulin separately

Procedure	Amount of serum ml.	Insulin effect micromoles (glucose equiv.) per gm. of tissue Mean±S.E.M.
Pre-exposure of diaphragm to serum	0.2	0.71±2.04 (3)*
Pre-exposure of diaphragm to insulin	0.2	1.15±0.46 (3)

( )* Number of determinations

TABLE 7

Failure of serum from insulin resistant diabetic acidosis patient to inhibit binding of I^{131}-insulin to rat diaphragm

Source of serum	Number of experiments	Counts/min. of I^{131} bound/gm. of diaphragm using I^{131}-insulin±S.E.M.
Normal	6	2,683±204
Case VII	6	2,758±144

TABLE 8

Absence of insulinase activity in insulin antagonist serum

Material	Amount ml.	% I^{131} from insulin-I^{131} in trichloroacetic acid supernatant after 60 min. incubation
Liver homogenate	0.5	57
Insulin antagonist serum	0.2	0
Normal serum	0.2	0

antagonist serum or normal serum was used.

The mechanism of action of the insulin antagonist found during diabetic acidosis cannot be attributed to any insulinase activity (table 8). Furthermore, insulinase has never been reported in the serum.[15] Since insulinase has been found in muscle,[15] it is conceivable, but unlikely, that the antagonist somehow augments or accelerates the degradation of insulin by this enzyme.

SUMMARY

The serum insulin antagonist which occurs during diabetic acidosis has been further characterized. It does not seem to be related to growth hormone secretion. In two cases studied, the antagonist was no longer demonstrable in the serum six to nine hours after the onset of insulin therapy. The activity was nondialyzable and migrated electrophoretically with the alpha-globulin fraction of the serum proteins. Pre-exposure of the rat hemidiaphragm to insulin or antagonist serum did not abolish the inhibition. The factor is not a lipoprotein and is inactivated by heating to 100° C for four minutes. It does not compete with insulin for binding sites on the diaphragm, nor does it prevent the binding of insulin by the diaphragm. It is devoid of insulinase activity.

It is postulated that the antagonist is bound rapidly and firmly to the diaphragm independent of insulin and then interacts with insulin to abolish its effect.

SUMMARIO IN INTERLINGUA

Observationes in Re le Natura del Antagonista Humoral a Insulina, Que Es Associate Con Acidosis Diabetic

Le antagonista seral a insulina que occurre in acidosis diabetic es describite plus detaliatemente. Il pare que illo non es relationate al secretion de hormon de crescentia. In duo del casos studiate, le antagonista non esseva demonstrabile in le sero sex a nove horas post le initiation del therapia a insulina. Le activitate del antagonista non esseva dialysabile e migrava electrophoreticamente con le fraction globulina alpha del proteinas seral. Pre-exposition del hemidiaphragma del ratto a insulina o a sero con antagonista non aboliva le effecto inhibitori. Le factor in question non es un lipoproteina; illo es inactivate per calefaction a 100 C durante quatro minutas. Illo non rivalisa con insulina in le occupation de sitos ligatori super le diaphragma e non preveni le ligation de insulina per le diaphragma. Illo possede nulle activitate de insulinase.

Es postulate que le antagonista es ligate rapidemente e firmemente al diaphragma sin dependentia de insulina e que postea illo interage con insulina, aboliente su effecto.

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DISCUSSION

FRANCIS D. W. LUKENS, M.D., (*Philadelphia*): The presentations by Dr. Colwell and Dr. Field provide a valuable up-to-date summary of the two principal components of insulin antagonism or insulin resistance, using those words in the broad sense. There certainly are immunologic forms of insulin resistance. Dr. Colwell and his discussers made that quite clear.

There is certainly some form of physiological or other antagonism, as Dr. Field brought out so well. Dr. Field demonstrated two things which should be correlated with what Dr. Colwell said, and I try to do so in this fashion. Dr. Field showed that two patients who had never been treated with insulin had insulin inhibitor. They had not developed antibodies to any exogenous product. Secondly, he showed that this inhibitor was no longer present after recovery from acidosis, a matter of hours, and most antibodies don't disappear that fast. Since I am not an immunologist, this is subject to correction.

There is one more thing. I think that Dr. Field said that he thought the pituitary and adrenal cortex did not have anything to do with this.

JAMES B. FIELD, M.D.: They weren't the main factors responsible for it.

DR. LUKENS: One of the difficulties is that these studies are insulin assays, whether the operated mouse or the rat diaphragm is used. They are not the direct chemical measurement of insulin. Now, an assay measures effective insulin, and effective insulin may be a trace of insulin with no inhibitor or it may be an enormous amount of insulin still with some inhibitor. That makes it difficult to differentiate as to what is happening at certain times. Dr. Vallance-Owen has worked with animals in which these glands have been removed. He finds the depancreatized animal has zero insulin by his method and considerable inhibitor. A Houssay animal (minus the pancreas and pituitary) has zero insulin and no inhibitor, so I think we must keep an open mind as to what any endocrine gland is doing. In addition the methods of assay have differed in different laboratories.

Thus, in Dr. Colwell's work, mice were used for assay. In Dr. Field's work glycogen deposition in the rat diaphragm was used, whereas Dr. Vallance-Owen is using the uptake of glucose from the medium by the rat diaphragm.

These technical differences must be resolved and understood before final interpretation is made, but I would like to congratulate both of these workers on a really marvelous presentation.

ROBERT WM. WEIGER, M.D., (*Chicago*): I would like to compliment Dr. Field on an excellent piece of work, and to ask a question prefaced by a comment.

P. G. Scheurlen in Germany has reported that in severe and untreated diabetes, he has observed an elevation of the alpha-2 globulin returned to normal after corrective treatment with insulin. This abnormal elevation was demonstrated electrophoretically by Scheurlen.

What were the alpha globulin contents of your patients' sera during the insulin resistant phases? Were there any quantitative changes in the alpha globulin contents of the sera after the patients had gone from the resistant to the nonresistant phase?

GARFIELD G. DUNCAN, M.D., (*Philadelphia*): I am sure that the workers doing the fundamental work would agree we shouldn't give up any simple clinical means of studying the return of sensitivity to insulin in patients in diabetic coma, until they have a better method available for clinical work.

Dr. Field stressed that there was no correlation of the antagonist with CO_2 combining power and the *p*H of the blood. He didn't mention whether or not there was any correlation with the degree of ketonemia, however. If I understood the slides correctly, there were two patients in diabetic coma who presented no antagonists in the serum. If these cases were in diabetic coma, then a study of the decreasing degree of ketonemia would be

a reliable means of detecting the return to sensitivity to insulin in patients who have no antagonist.

I would like to ask Dr. Field if he has had an opportunity in this splendid work of his to study a parallel between the antagonist and the degree of ketonemia because in my experience as the ketonemia subsides in the treatment for diabetic coma, insulin sensitivity returns without fail.

GEORGE M. GUEST, M.D., (*Cincinnati*): I, too, should like to pay compliments to this beautiful piece of work. Since Dr. Field mentioned the studies that Dr. Mackler and I reported on the inhibiting effects of acidosis on insulin action, I should like to elaborate this point.

As explained in our reports, we employed ammonium chloride infusions in normal dogs to produce acidosis in order to study the effect of acidosis, per se, apart from other factors of ketonemic acidosis in diabetic subjects. The results indicated that low *p*H is a critical factor inhibiting the blood-sugar lowering effect of insulin and at the same time inhibiting the decreases of potassium and inorganic phosphorus in the blood that normally accompany the fall of blood sugar after insulin. It should be noted also that immediately after acidosis was corrected by sodium bicarbonate these dogs responded normally to test doses of insulin, showing that the acidotic state left no lasting inhibitory aftereffect on insulin action. Thus, it must be borne in mind that the effect of acidosis, low *p*H of the blood, in inhibiting insulin action, is only one of many factors in the metabolic derangements that may lead to insulin resistance in the patient with diabetic coma. Ketonemia, stressed by Dr. Duncan, is closely correlated with the severity of acidosis in diabetic coma, another one of many closely interrelated factors in the vicious circle of mutually aggravating disturbances.

RACHMIEL LEVINE, M.D., (*Chicago*): I was much interested in this presentation and the one preceding it, since they begin to throw light on the various phases of insulin antagonism and resistance. It struck me that the two patients mentioned by Dr. Field, who despite acidosis did not show insulin resistance, were just the patients who had high corticosteroid levels in their blood; as if they were spontaneously treated with ACTH.

I wonder whether the common denominator is the outpouring by the liver, under certain conditions, of many species of proteins. In one case, alpha globulins, containing a factor which combines with insulin; in another case gamma globulins, which are immunologically related to insulin. It may be that ACTH and the steroids nonspecifically depress the production and release of all such protein fractions.

Have you done any studies on the corticosteroid level of patients who did show the insulin antagonist, as contrasted with those who didn't? Since your factor is not trypsin-sensitive and is attached to the alpha globulins, is there any possibility that it is allied to the protamines since these substances are good "binders" of insulin?

DR. FIELD: I would like to thank the discussers for their discussions. In regard to Dr. Weiger's question, we have not measured any alpha 1 or alpha 2 globulin levels in patients during diabetic acidosis so we have no information other than the report by Scheurlen (Scheurlen, P. G. Serum protein changes in diabetes mellitus. Klin. Wchnschr. 33:198-205, March 1, 1955) from Germany, that these protein fractions are elevated.

In regard to Dr. Duncan's question, we have had only one occasion to attempt to correlate the degree of ketonemia and insulin resistance. In this patient there was a good correlation.

We are hopefully waiting for Dr. Duncan to supply us with some specimens in which he has done the qualitative acetone test without telling us the results, and then we can do our assay, and then see if there is any correlation or not. But it certainly might be that this would be an excellent way of telling when a patient first is admitted as to whether or not large doses of insulin will be required.

In answer to Dr. Levine's question, we have not measured the steroid levels in the patients who have insulin antagonist.

One of the difficulties in this problem has been procuring enough serum to test for antagonist, and if there is antagonist, to have enough serum left to do other studies. So far we have not felt we could spare the amount of serum necessary for measuring blood steroids. However, if we do have the occasion where we have enough serum from a patient, I think that would be a very worth-while thing to do.

The possibility of this being protamine or some other substance that binds insulin certainly exists. The fact that the antagonist does not prevent insulin from being bound to the tissue, and the antagonist also is effective whether the diaphragm is exposed to it or insulin first would tend to indicate if there is any binding, it is within the diaphragm itself rather than in the medium before the diaphragm is exposed to insulin.