Failure of Guinea Pig Antibody to Beef Insulin to Neutralize Coypu (Nutria) Insulin

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SUMMARY

Guinea pig insulin is the only mammalian insulin previously known to be nonneutralizable by antibody to beef insulin (Canad. J. Physiol. Pharm. *43*:373, 1965). A second nonneutralizable mammalian insulin, that of the coypu (nutria, or myocastor coypus), has been identified. Coypu pancreas yielded 1.95 U. extractable insulin per gram. An amount of guinea pig antibody to beef insulin sufficient to neutralize twenty-five times as much beef insulin had no effect on the increase in glycogen content produced by pancreatic or serum coypu insulin in the mouse hemidiaphragm in vitro. Both 50 mU. coypu insulin alone and 50 mU. coypu insulin plus 150 mU. antibody to beef insulin when injected into mice produced as many convulsions and lowered the blood glucose to the same mean level as did 50 mU. beef insulin alone (12 mg./100 ml.). Mice injected with 50 mU. beef insulin plus 150 mU. antibody did not convulse and had a mean blood glucose of 108 mg./100 ml. Coypu pancreatic extract cross-reacted with guinea pig antiserum to beef insulin in the passive cutaneous anaphylaxsis test. DIABETES *17*:8-12, January, 1968.

The only mammalian insulin previously known to be nonneutralizable by antibody to beef insulin is that of the guinea pig.[1-10] The following observations indicate that there is a second mammalian insulin that is not neutralized by guinea pig antibody to beef insulin, namely that of the coypu (nutria, or myocastor coypus).

MATERIALS AND METHODS

Preparation and assay of coypu pancreatic and serum insulin activity

A fed 6.2 kg. male coypu was anesthetized with Nembutal and exsanguinated through a carotid cannula. The serum was separated by centrifugation and frozen. The pancreas, which weighed 10 gm., was removed surgically and frozen between slabs of solid carbon dioxide. Later the pancreas was extracted with acid alcohol and prepared for testing by the dialysis method.[11] Both native serum and serum treated with acid alcohol and dialyzed were also tested.

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The pancreatic extract, native serum, and acid-alcoholic-treated dialyzed serum were assayed for their insulin activity content by the mouse hemidiaphragm insulin assay.[2,3] The pancreatic extract contained 1.95 U. insulin per gram, the native serum contained 0.36 mU. insulin activity per ml., and the treated serum contained 1.85 mU. insulin activity per ml.

Preparation of guinea pig sera containing antibodies to beef insulin

Guinea pig antibodies to beef insulin were prepared by the method of Moloney and Coval,[6] and their neutralizing potency determined as previously.[9] Each milliliter of the immune serum used in these studies was capable of neutralizing 600 milliunits (mU.) of beef insulin.

Determination of insulin activity in solutions containing coypu insulin with and without guinea pig antibody to beef insulin

The hormonal activity of beef and coypu insulins in the absence and presence of guinea pig antibody to beef insulin was compared by measuring three insulin-responsive metabolic parameters: (1) increase in glycogen content of mouse hemidiaphragm in vitro, (2) convulsions in mice, and (3) blood glucose lowering in mice.

(1) *Mouse hemidiaphragm method.* Aliquots of extracts of coypu pancreas containing 0.1 mU. and 0.2 mU. coypu insulin were mixed with aliquots of guinea pig serum containing antibody in amounts sufficient to neutralize 2.5 mU. beef insulin and tested.

Aliquots of native coypu serum containing 0.09 mU. insulin activity (NSIA) and aliquots of acid-alcoholic-treated coypu serum containing 0.14 mU. insulin activity (TSIA) were mixed with aliquots of guinea pig serum containing antibody in amounts sufficient to neutralize 10 mU. beef insulin and tested.

The mean relative glycogen contents of hemidiaphragms incubated in buffer, in a solution of beef insulin with and without antibody, and in a solution of coypu insulin with and without antibody were determined.

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(2) *Mouse convulsion method.* Twenty-gram Canadian Breeding Laboratory albino male mice were deprived of food but not water for four hours and then randomly assigned to one of four test groups. One group (eleven mice) was injected subcutaneously with 50 mU. beef insulin alone; one group (eleven mice) was injected with 50 mU. beef insulin plus 150 mU. antibody to beef insulin; one group (twelve mice) was injected with 50 mU. coypu insulin alone; one group (eleven mice) was injected with 50 mU. coypu insulin plus 150 mU. antibody to beef insulin. Immediately after injection, the mice were grouped and placed in compartments of a constant temperature bath at 38° C. The mice were observed for convulsions for a period of ninety minutes.

(3) *Mouse blood glucose lowering method.* Mice that convulsed were immediately removed from the compartment of the bath, decapitated with scissors, and blood was collected for glucose determination by the glucose oxidase method.[12] Those that did not convulse were decapitated ninety minutes after injection and blood was collected for glucose determinations.

Test of cross-reactivity between coypu pancreatic extracts and beef insulin antiserum in the passive cutaneous anaphylaxis reaction in guinea pigs

Normal depilated guinea pigs were injected intradermally with 0.1 ml. aliquots of various sera from beef-insulin-immune guinea pigs. The guinea pigs were challenged intravenously twenty to twenty-two hours later with 20 mU. coypu insulin in 1 ml. saline containing 5 mg. pontamine sky blue 6 BX.[13] The appearance of a blue spot at the site of intradermal injection of antiserum within ten to twenty minutes after intravenous injection of a highly purified insulin preparation indicates that an antibody-insulin reaction has taken place. Such a reaction causes local tissue anaphylaxis and increases vascular permeability, and this in turn causes leakage of pontamine sky blue into the skin.

Failure of Guinea Pig Antibody to Beef Insulin to Neutralize Insulin Activity of Coypu Pancreatic Extract

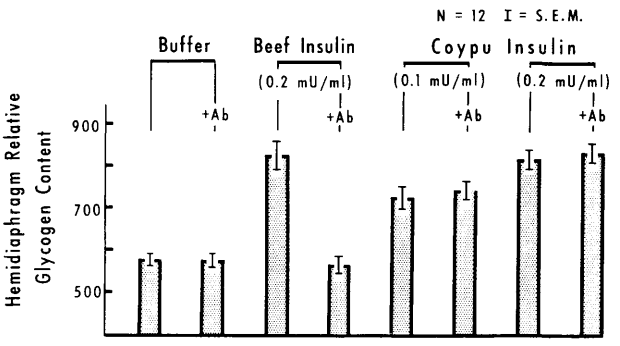

FIG. 1. Each incubation tube (designated +Ab) contained sufficient guinea pig antibody to beef insulin per ml. to neutralize 2.5 mU. beef insulin. S.E.M. = standard error of the mean.

EXPERIMENTAL RESULTS

Guinea pig antibody to beef insulin completely neutralized the glycogen-content-increasing effect of beef insulin; antibody to beef insulin did not alter the glyco-

Failure of Guinea Pig Antibody to Beef Insulin to Neutralize Insulin Activity of Coypu Serum

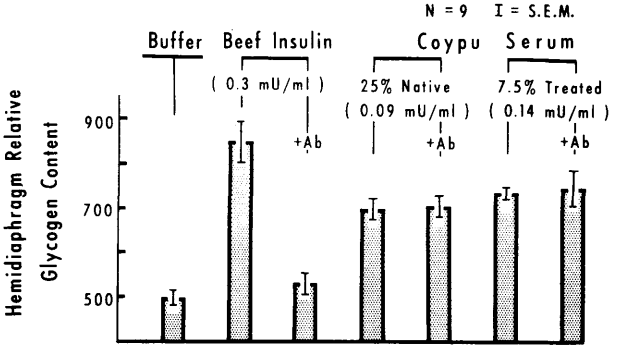

FIG. 2. Each incubation tube (designated +Ab) contained sufficient guinea pig antibody to beef insulin per ml. to neutralize 10 mU. beef insulin. S.E.M. = standard error of the mean.

TABLE 1

Convulsions and terminal blood glucose levels in mice after injection of beef and coypu insulins with and without antibody to beef insulin

	50 mU. beef insulin	50 mU. beef insulin plus 150 mU. beef insulin antibody	50 mU. coypu insulin	50 mU. coypu insulin plus 150 mU. beef insulin antibody
No. of mice that convulsed	10 of 11	0 of 11	11 of 12	11 of 11
Terminal blood glucose (mg./100 ml.)	Mean = 12 Range = 8 — 20	Mean = 108 Range = 87 — 126	Mean = 12 Range = 7 — 28	Mean = 12 Range = 7 — 14

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gen-content-increasing effect of pancreatic coypu insulin (figure 1). Table 1 compares the effects of beef insulin and pancreatic coypu insulin injected with and without antibody to the effects of an equivalent amount of beef insulin alone in producing convulsions and in lowering the blood glucose of mice. Both coypu insulin alone and coypu insulin plus antibody to beef insulin when injected into mice produced as many convulsions and lowered the blood glucose to the same mean level as did beef insulin alone. Mice injected with beef insulin plus antibody to beef insulin did not convulse and had normal blood glucose levels. Antibody to beef insulin altered neither the glycogen-content-increasing effect of coypu native serum nor the glycogen-content-increasing effect of coypu acid-alcoholic-treated dialyzed serum (figure 2).

In the PCA test, twenty milliunits of coypu insulin intravenously gave a moderate to strong reaction with beef insulin antiserum in each of four guinea pigs. The average diameter of the blue spots that appeared was 22 mm., with a range of diameters between 16 mm. and 30 mm.

DISCUSSION

Up to the present time, the hormonal activity of all mammalian insulins tested, with the exception of that of the guinea pig, have been found to be neutralizable by the serum of guinea pigs immune to beef insulin.[1-10] The neutralizable pancreatic insulins include beef, sheep, pig, horse, whale, dog, cat, rabbit, rat, Chinese hamster, mouse, monkey, and man. The insulin activity of native serum from dog and man are also essentially completely neutralizable[1,2] when tested by the mouse hemidiaphragm assay. Endogenous insulins of the mouse, rat, cat, rabbit, dog, sheep, and pig are also neutralizable by intravenously or intraperitoneally injected guinea pig antibodies to beef insulin. Antibodies of this type have been used to produce an acute endogenous insulin deficiency and experimental diabetes in the indicated animals.[6,14] Mice and rabbits repeatedly injected with beef insulin may develop beef insulin antibodies and become hyperglycemic, apparently due to cross-reactivity of the animal's endogenous insulin with antibodies to beef insulin. In some of these animals a break in immunologic tolerance to endogenous insulin occurs, and antibodies to endogenous insulin and presumably autoimmune-induced destructive lesions of the beta cells develop.[5,15] Guinea pigs injected with beef insulin develop a high titer of beef insulin-neutralizing-antibodies in their serum but do not become hyperglycemic,[10] and no destructive lesions of the beta cells have been described in such animals. Injection of extracted pancreatic guinea pig insulin into guinea pigs immune to beef insulin lowers the blood glucose to the same level as does injection of the same amount of guinea pig insulin into nonimmune guinea pigs.[10]

The above reports indicate that cross-reactivity of exogenous and endogenous guinea pig insulin with beef insulin antibody is undetectable in the hormone neutralization tests that have been carried out. The results of the current study indicate that the hormonal activity of pancreatic coypu insulin as measured by the mouse convulsion test and the mouse blood-glucose-lowering test is not altered by a three-fold excess of antibody to beef insulin, nor is it altered by a twenty-five-fold excess of antibody when glycogen synthesis by the mouse hemidiaphragm is the metabolic parameter of insulin action used. The hormonal activity of coypu native serum in the mouse diaphragm test is not altered by the presence of a 100-fold excess of beef insulin antibody, and the hormonal activity of coypu treated serum in the mouse diaphragm test is not altered by the presence of a seventy-fold excess of beef insulin antibody.

Wilson[16] has recently reviewed the hormonal behavior of insulins from different species in the presence of guinea pig-antibody to beef insulin. All mammalian insulins tested up to that time, except that of the guinea pig, cross-reacted in neutralization tests; chicken, frog, bowfin, pike, sucker, and sunfish insulins also cross-reacted. That cross-section was less frequent in lower order species was indicated by the fact that sculpin, catfish, cod, pollock, bonito, tuna, dogfish, lemon shark, and hogfish insulins reacted poorly or not at all.

All mammalian insulins whose structures have been studied, with the exception of those of the guinea pig and the coypu, have amino acid sequences that differ only slightly or moderately from the amino acid sequence of beef insulin. Sheep insulin has one different amino acid residue; pig, whale, and dog insulins have two different amino acid residues; horse, rabbit, and human insulins have three different amino acid residues. Rat insulin has six different amino acid residues, the same number of differences that are present in an avian insulin, that of the chicken. By contrast, guinea pig insulin has eighteen amino acid residues that differ from those of beef insulin. Coypu insulin has approximately the same number of amino acid residues that differ from those of beef insulin.[17] Interestingly enough, it appears that about fourteen amino acid residues of coypu insulin differ from those of guinea pig insulin.[17] The C-terminal

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amino acid in beef insulin is asparagine at position A-21. The A-chain of coypu insulin contains twenty-two amino acid residues, with asparagine at position A-21 and aspartic acid at position A-22.[18] It has been suggested that the C-terminal portion of the A chain is of particular importance to the biological and immunological activity of insulin.[19] Insulins from lower order phylogenetic species that have been studied also differ markedly in amino acid sequence from beef insulin (eleven differences in bonito insulin, sixteen differences in cod insulin, and fifteen to seventeen differences in toad-fish insulin). It is possible that the structure of insulins from lower order animals that cross-react with antibody to beef insulin in neutralization tests resembles the structure of beef insulin more closely than those that do not cross-react. This problem cannot be resolved until the amino acid sequences of these insulins are known.

Presently available evidence thus indicates that the primary structure of insulins that cross-react with beef insulin antibodies in neutralization tests is not greatly different from the primary structure of beef insulin. This in turn suggests that relatively large portions of the surface configuration of such insulin molecules are available for combination with beef insulin antibody. It seems likely that neutralization occurs most readily when there is a good fit between insulin and antibody, and when there is a slow rate of dissociation of the insulin-antibody complex. Since both guinea pig and coypu insulins differ markedly in structure from beef insulin, it seems likely that there is a poor fit between these insulins and antibody to beef insulin. This probably accounts for the failure of antibody to beef insulin to alter the hormonal activity of either guinea pig or coypu insulin.

Insulin antibodies are heterogenous, with both gamma A and gamma G antibodies being present in the serum of guinea pigs immune to beef insulin.[20] It is not known which fraction contains the antibodies that are capable of neutralizing the hormonal effects of insulin. Anaphylactic antibodies are of the gamma A type, hemagglutinating and complement-fixing antibodies are of the gamma G type, and precipitating antibodies are of both types.[21]

Both the A and B chains of insulin, neither of which possesses hormonal activity, can combine with gamma A insulin antibodies and give an anaphylactic reaction.[13] A positive PCA test depends on the combination of relatively small haptenic sites with the gamma A type antibodies, and cross-reactivity of different insulins in the PCA test may occur in the presence of small haptenic similarities. The positive PCA test in this study suggests that coypu insulin and beef insulin share at least one small cross-reacting haptenic site. Lack of correlation between the neutralization tests and the PCA test suggests one of two possibilities: (1) Neutralizing antibodies may not be of the gamma A type, thus titers of anaphylaxis-producing and insulin-neutralizing antibodies may vary independently. (2) If neutralizing antibodies are of the gamma A type, the failure of the beef insulin antibody to alter hormonal activity of coypu insulin may be due to (a) the fact that the antibody combines with a small site on the insulin molecule and this site is not essential to biological activity, or (b) there may be rapid dissociation of the antibody-insulin complex.

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Dietary Carbohydrate and Liver Lipids

While many efforts directed to determining the relationship of lipids to heart disease have involved diets containing various types of fats, there are considerable data which indicate that the level of lipids in various tissues is markedly affected by carbohydrate. It has been suggested that the level of carbohydrate is related to the development of atherosclerosis. There is a marked increase in the concentration of cholesterol and lipoproteins in the blood when diets high in carbohydrate are fed to experimental animals (O. W. Portman, E. Y. Lawry, and D. Bruno, Proc. Soc. Exp. Biol. Med. *91*:321, 1956).

Studies of dietary trends in various population groups have suggested that as more refined carbohydrates are added to the diet and the more complex carbohydrates, such as starches, eliminated, there tends to be an increased incidence of coronary heart disease. Unfortunately, other items in the diet also change, so that the amount of protein and fat varies as much as, or more than, the type of carbohydrate. However, changes in the source and amount of carbohydrate can lead to changes in the quantity and quality of lipids present in tissues, and may be in some way responsible for an increased frequency of arteriosclerotic heart disease (Nutrition Reviews *21*:228, 1963).

The relationship of carbohydrate to protein requirements has also been studied. It has been claimed by some workers that the complex carbohydrate can lead to an increased synthesis of essential amino acids by the intestinal microflora (Nutrition Reviews *17*:107, 1959). However, detailed studies indicate that such effects are minimal, and the suggestion has been made that complex carbohydrates may have an effect on stomach volume and water retention.

In general, low protein-high carbohydrate diets lead to an accumulation of lipid in the liver. When such diets were fed to rabbits, there was a gradual weight loss, and finally the animals died (Nutrition Reviews 20:211, 1962). In these diets, containing only 7 per cent protein and 68 per cent carbohydrate, there was a difference in the development of lipid, depending upon whether the source of carbohydrate was sucrose or starch. Sucrose led to the formation of more extensive fatty livers and also increased the quantity of blood lipids, including sterol esters, triglycerides, and phospholipids.

While atherosclerosis seems to be less prevalent in sections of the world where diets rich in complex carbohydrate are eaten, hepatic cirrhosis is often more prevalent in some of these developing areas, such as Africa. It has been suggested that low protein-high carbohydrate diets, plus infection, (often schistosomiasis) are responsible for the high incidence of cirrhosis.

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