Induction of Hyperglycemia with Insulin Antibodies to B-Chain Determinants

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SUMMARY

Insulin antibodies measured by a radioimmune method (AB_R) are significantly better inducers of hyperglycemia than are insulin antibodies measured by an immune hemolysis method (AB_H) when injected intraperitoneally into mice. The ability to induce hyperglycemia by an insulin antiserum can be predicted by the titer of AB_R measured. AB_R interact in vitro with determinants severely perturbed on nickel-insulin, partially perturbed on proinsulin and desasparagine-desalanine insulin, and unaffected on zinc-insulin or zinc-free monocomponent insulin. AB_H, on the other hand, interact in vitro with determinants severely perturbed on proinsulin and desasparagine-desalanine insulin but stabilized on nickel-insulin and zinc-insulin. Since the connecting peptide of proinsulin is probably in apposition to the A-chain residues on the solvent surface, the more effective reaction of proinsulin with AB_R than with AB_H is submitted as evidence that AB_R are directed toward residues on the B-chain surface of insulin. Because AB_R are more effective inducers of hyperglycemia than are AB_H, it is proposed that the degree of hyperglycemia induced by antibodies in vivo is a result of interactions with determinants on the B-chain surface of insulin. These results support the possibility that insulin in vivo is more accessible for interaction with antibodies directed to the B-chain of insulin. It is also possible that AB_R, which are directed to B-chain determinants, are of higher affinity than is the affinity between insulin and receptors or that the active site of insulin for maintaining euglycemia includes the B-chain surface residues. **DIABETES 29:383–390, May 1980.**

Presented in abstract form at the annual meeting of the Federation of American Societies for Experimental Biology held at Dallas in April, 1979, and the Second International Insulin Symposium held at Aachen in September, 1979.
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Received for publication 30 August 1979 and in revised form 9 January 1980.

Antisera used to measure insulin in immunoassay systems are composed of several populations of antibodies. Each population is in turn directed toward one of several determinants on the insulin molecule. Previous studies demonstrated that insulin derivatives with perturbed A-chain determinants exhibited higher relative immunologic activity in a radioimmune inhibition assay than in an immune hemolysis-inhibition assay.[1,2] From these results and the knowledge of the structure of insulin,[3] it was possible to propose that B-chain surface residues form determinants which interact with the antibodies operative in the radioimmune inhibition assay (AB_R).* In addition, it was possible to obtain evidence that the connecting peptide of proinsulin is in apposition to the surface residues of the A-chain of insulin and that antibodies operative in the immune hemolysis inhibition assay (AB_H) interact with determinants formed by A-chain surface residues.

The induction of hyperglycemia by guinea pig insulin antiserum injected into mice, rats, rabbits, cats, dogs, pigs, and sheep has been explained as being caused by the binding of the antibodies with endogenous insulin in the circulation.[4–6] The experiments presented in this report were designed to determine whether insulin in vivo—in circulation, on the cell membrane, or both—is oriented in a specific manner or is freely accessible to binding with all antibodies. Pools of insulin antisera were formed with antisera selected on the basis of the titer of AB_R or AB_H. By performing inhibition studies with five derivatives of insulin, additional evidence was obtained to further support the earlier studies[1,2] that AB_R are directed toward determinants formed by B-chain surface residues, whereas AB_H are directed toward determinants formed by A-chain surface residues.

When antisera were injected intraperitoneally into mice, a

* Abbreviations used in this paper are: AB_R, antibodies measured by the radioimmune system; AB_H, antibodies measured by the immune hemolysis system; AP, antiserum pool; Zn-insulin, zinc-insulin; MC-insulin, zinc-free monocomponent insulin; Ni-insulin, nickel insulin; DAA-insulin, desasparagine-desalanine insulin; RIIA, radioimmune inhibition assay; IHIA, immune hemolysis inhibition assay.

qualitatively superior ability to induce hyperglycemia by antisera with high titers of AB_R was observed as compared with the hyperglycemia induced by an antiserum pool with high titers of Ab_H. It is therefore possible to predict the ability of antisera to induce hyperglycemia by determining the level of AB_R present. It is proposed that the antibody binding to the B-chain determinants is of a higher affinity, is more readily accessible in vivo, or that the B-chain surface of insulin expresses its biologic activity.

MATERIALS AND METHODS

Preparation and characterization of insulin antiserum pools. Mongrel outbred guinea pigs were immunized with either monocomponent bovine insulin, fibrillar bovine insulin, or 10 times recrystallized Zn-bovine insulin (Novo Company) by a method previously described.[7] Each guinea pig received 0.125 mg of antigen in 0.20 ml of complete Freund's adjuvant in each paw pad. Fourteen days later, three equally spaced booster injections were given intradermally during a 7-day period. Each booster injection contained 0.1 mg of the antigen in 0.1 ml of buffered saline. Seven days later this procedure was repeated. Ten days after the booster injection, 1-ml blood samples were taken from the hind leg of each animal and titrated. When satisfactory antibody titers were obtained, the guinea pigs were exsanguinated immediately. Blood samples were allowed to clot at room temperature for 1 h and then overnight at 4°. The next day the serum was separated by centrifugation and stored at −80°.

The titer of AB_R and AB_H of the antiserum from each animal was measured by radioimmune titration and immune hemolysis titration,[2,8] respectively. The radioimmune titer is the dilution of antiserum of which 0.1 ml will bind 50% of 2×10^4 cpm of ^{125}I-monoiodoinsulin[9] (approximately 0.059 ng, sp act = 200 mci/mg) in a final volume of 0.3 ml. The immune hemolysis titer is the dilution of antiserum of which 0.1 ml will hemolyze 50% of 8.7×10^6 sheep red blood cells coated with Zn-insulin[10] in a final volume of 0.4 ml. In both titrations, antiserum and the insulin indicator were incubated for 1 h at 4°C. In the radioimmune titration, dextran-coated charcoal was added after the initial incubation to separate bound from free ^{125}I-monoiodoinsulin as previously described by Herbert.[11] In the immune hemolysis titration, excess complement was added after the initial incubation, the mixture was then incubated at 37° for 30 min, and the degree of specific immune hemolysis was determined as previously described.[7,10]

Three antiserum pools (AP) were prepared according to the radioimmune titers and immune hemolysis titers of the component insulin antisera. AP1 and AP2 were pools of antisera with high radioimmune titers and low immune hemolysis titers. AP3 was a pool containing antisera with low radioimmune titers and high immune hemolysis titers. AP1 and AP2 had high radioimmune titers and low immune hemolysis titers. AP3 had a low radioimmune titer and a high immune hemolysis titer (Table 1).

Insulin derivatives. MC-insulin was prepared from twice recrystallized bovine insulin (Elanco Co.) by chelation with EDTA and purification on a DEAE-Sephadex column equilibrated with 0.01M Tris, 0.01M glycine, 0.08M NaCl, and 7M urea buffer, pH 9.[9,12] The MC-insulin migrated as a single band in 15% acrylamide slab gel electrophoresis, indicating homogeneity and less than 2% contamination. The zinc content of the MC-insulin was analyzed by atomic absorption and was found to be less than one atom of zinc per 38 insulin molecules (UCIMC Laboratories, Orange, California). A single preparation of MC-insulin was used in the inhibition studies. In addition, this same preparation was used as the starter to prepare the Zn-insulin and Ni-insulin used in the inhibition studies.

TABLE 1
Radioimmune titer and immune hemolysis titer for each insulin antiserum pool (AP)

AP	Radioimmune titer*	Immune hemolysis titer†
AP 1	11,500	1,700
AP 2	9,000	405
AP 3	1,200	5,110

* Radioimmune titer is the antiserum dilution of which 0.1 ml will bind 50% of 2×10^4 cpm of ^{125}I-monoiodoinsulin (approximately 0.059 ng, sp act = 200 mCi/mg) in a final volume of 0.3 ml.
† Immune hemolysis titer is the insulin antiserum dilution of which 0.1 ml will hemolyze 50% of 8.7×10^6 sheep red blood cells coated with Zn-insulin aggregates in a final volume of 0.4 ml.

Purified bovine proinsulin was a gift from Dr. Jorgen Schlichtkrull of the Novo Company, Dr. Ron Chance of the Eli Lilly Company, and Dr. Don Steiner, Dept. of Biochemistry, U. of Chicago. The three proinsulins were not significantly different from each other immunologically or by 15% acrylamide slab gel electrophoresis.

DAA-insulin was prepared by the exhaustive digestion of insulin with carboxypeptidase A[13] and was purified by column chromatography.[7]

MC-insulin, proinsulin, and DAA-insulin were all dissolved in 0.01N NaOH and were immediately diluted to approximately 1.74 μM in an equal volume of 0.22M phosphate buffer. The concentration was adjusted with sample buffer to exactly 1.74 μM spectrophotometrically (molar absorptivity at 276 nm = 5950).[2] All stock insulin solutions were stored at 4°C for no longer than 2 wk. The concentration was confirmed by molar absorptivity when used.

Zn-insulin and Ni-insulin were freshly prepared from MC-insulin stock. To 0.1 ml of 1 mg/ml (1.74 μM) MC-insulin stock, 0.02 ml of 0.523 mM $ZnSO_4$ or $Ni(NO_3)_2$ was added. The resulting 0.833 mg/ml (1.45 μM) Ni- and Zn-insulin solutions (approximately 0.6 atoms Zn or Ni per molecule of insulin) were then shaken at 175 rpm at 4°C for exactly 1 h. The Ni- and Zn-insulin solutions were used exactly 1 h after being diluted to the appropriate concentrations in either of the two immune inhibition assays.

Acrylamide slab gel electrophoresis. The acrylamide slab gel electrophoresis was a modification of the method of Laemmli.[14] An 11-cm-slab gel, containing 15% acrylamide and 0.4% N,N′-bis-methylene acrylamide, was used. Polymerizing agents employed were tetramethylethylenediamine and ammonium persulfate in a final concentration of 0.05%. Insulin (0.02 ml, 1.74 μM in 0.11M phosphate buffer, pH 7.4) and tracking dye (0.005 ml, 0.02% bromophenol blue and 50% glycerol) were run at 30 mA until tracking dye was 1 cm from the bottom of the gel. Staining and destaining were accomplished at 60°C. Staining solution was 0.1% coomassie blue (Sigma Co.), 7% acetic acid, and 10%

methanol. Destaining solution was 7% acetic acid and 10% methanol.

Immune inhibition assays. In the radioimmune inhibition assay (RIIA) and immune hemolysis inhibition assay (IHIA), a fixed dilution of insulin antiserum was reacted with a fixed concentration of the respective indicator insulin (^{125}I-monoiodoinsulin or Zn-insulin-coated sheep red blood cells) in the presence of varying concentrations of inhibitor.[2,8] The ability of a given insulin derivative to inhibit the binding of antibodies to indicator insulin in each assay system was measured.

For the RIIA and IHIA, each AP was diluted to its titer in the respective assay. For example, for AP1, the radioimmune titer was 11,500 and the immune hemolysis titer was 1,700. Therefore, in the RIIA, AP1 was diluted 1:11,500, while in the IHIA, AP1 was diluted 1:1,700. The amount of binding or lysis at these dilutions, when no inhibitor was added, was used as the maximum binding or lysis for the calculation of percent inhibition. The appropriately diluted antiserum (0.1 ml) was added to 0.1 ml of indicator insulin and 0.1 ml of the appropriate insulin derivative used as inhibitor. The assays were then run exactly as in the radioimmune or immune hemolysis titrations described above.

Measurement of diabetogenic activity. Male C57 B1/6 mice (Microbiological Associates), 8 to 12 wk of age, fed Purina mouse chow and water ad libitum, were injected intraperitoneally with one of the three antiserum pools (AP). Control groups received normal guinea pig serum. Groups of ten animals received one of four doses—0.05, 0.1, 0.2, or 0.4 ml of AP1, AP2, or AP3. Simultaneously, control groups of five or ten animals each received comparable doses of normal guinea pig serum.

Blood glucose was measured at 0, 15, 30, 45, 60, 90, 120, 180, and 240 min after injection. Blood samples (0.025 ml) were taken from the periorbital sinus, diluted 1:5 in distilled water, and glucose levels were determined by a glucose oxidase method with a glucose analyzer (YSI-model 23A). The diabetogenic activity was determined by calculating the difference between the area under the blood glucose vs. time curve for a given dose of a given AP and the area under the same curve for normal guinea pig serum and was expressed as mg glucose/dl × min during the four hours after injection.

The degree of correlation between diabetogenic activity and both radioimmune titer and immune hemolysis titer was calculated by the method of least squares for each of the antisera pools.

RESULTS

Binding characteristics of antibodies in the insulin antiserum pools. Inhibition studies in both immunoassay systems were performed with five insulin derivatives: MC-insulin, Zn-insulin, Ni-insulin, DAA-insulin, and proinsulin. Each antiserum pool was diluted to its respective titer for each immunoassay system. Therefore, the levels of antibodies in each AP equaled the amount of antibody that bound 50% of the ^{125}I-insulin in the radioimmune titration or that was capable of hemolyzing 50% of the Zn-insulin-coated red cells in the immune hemolysis titration. The amount of inhibitor (picomoles) required to inhibit 50% of the interaction between antibodies and the indicator (I^{125}-insulin or insulin-coated sheep red blood cells) in each of the respective assays was used to determine the relative degree of immunologic reactivity of each derivative.

The interaction of the five inhibitors with the insulin antibodies in each AP is presented in Figures 1 and 2 and Tables 2 and 3. Ni-insulin reacted poorest of the five derivatives with AB_R (Figure 1, Table 2). Approximately five times (20% cross reaction) as much Ni-insulin as MC-insulin was required to obtain 50% inhibition. Both DAA-insulin and proinsulin reacted about half as well as MC-insulin. Zn-insulin reacted as well as MC-insulin. It follows that the determinants on the insulin molecule that interact with AB_R are perturbed to the greatest degree on Ni-insulin, to a lesser degree on DAA-insulin and proinsulin, and not at all on Zn-insulin when compared with MC-insulin.

In contrast, Ni-insulin was the most reactive of the five inhibitors with AB_H (Figure 2, Table 3). An average of one fortieth as much Ni-insulin (40-fold greater reactivity) as MC-insulin was required for 50% inhibition. An enhanced reaction with Zn-insulin was also observed. Zn-insulin reacted approximately 10 times better than MC-insulin with AB_H. Proinsulin and DAA-insulin reacted poorest of the five inhibitors with AB_H, each reacting approximately one thirtieth as well as MC-insulin. The poor reactivity of proinsulin and DAA-insulin with AB_H suggests that the determinants with which they interact are perturbed to a greater degree on proinsulin and DAA-insulin than on the other three insulin derivatives tested.

The determinants on Ni-insulin with which the AB_R interact were found to be maximally perturbed. On the other hand, the determinants on Ni-insulin with which AB_H interact can best be described as more stable (most reactive) than these same determinants on any of the other derivatives tested. The determinants on Zn-insulin with which AB_R interact were not perturbed. The determinants on Zn-insulin with which AB_H interact were stabilized in a manner similar to Ni-insulin.

These observations are consistent with previous results.[2] The observations with Ni-insulin are new and are best explained as being caused by a marked perturbation of the B-chain surface determinants with which AB_R interact. Surprisingly and of interest was the augmented reactivity of Ni-insulin with AB_H, which is best explained by stabilization of the determinants on the A-chain surface (Figure 3).

The diabetogenic activity of insulin antiserum pools. The level and duration of the hyperglycemia induced was related to the radioimmune titer and the dose of AP injected. The plots of blood glucose vs. time for the 0.1-ml dose of each insulin antiserum pool are presented in Figure 4. The diabetogenic activity of the two insulin antiserum pools with high radioimmune but low immune hemolysis titers (AP1 and AP2) was qualitatively greater than that measured for the insulin antiserum pool with a low radioimmune titer and a high immune hemolysis titer (AP3). The calculated diabetogenic activities when 0.1 ml each of AP1 and AP2 were injected intraperitoneally were 36,713 and 30,433 (mg glucose/dl × min), respectively, compared with 6,721 when 0.1 ml of AP3 was injected. There was a slight difference in the hyperglycemia induced by AP1 and AP2. AP2 induced a steeper hyperglycemia, peaking at 60 min, with a quicker decline to normal levels, compared with AP1, where the

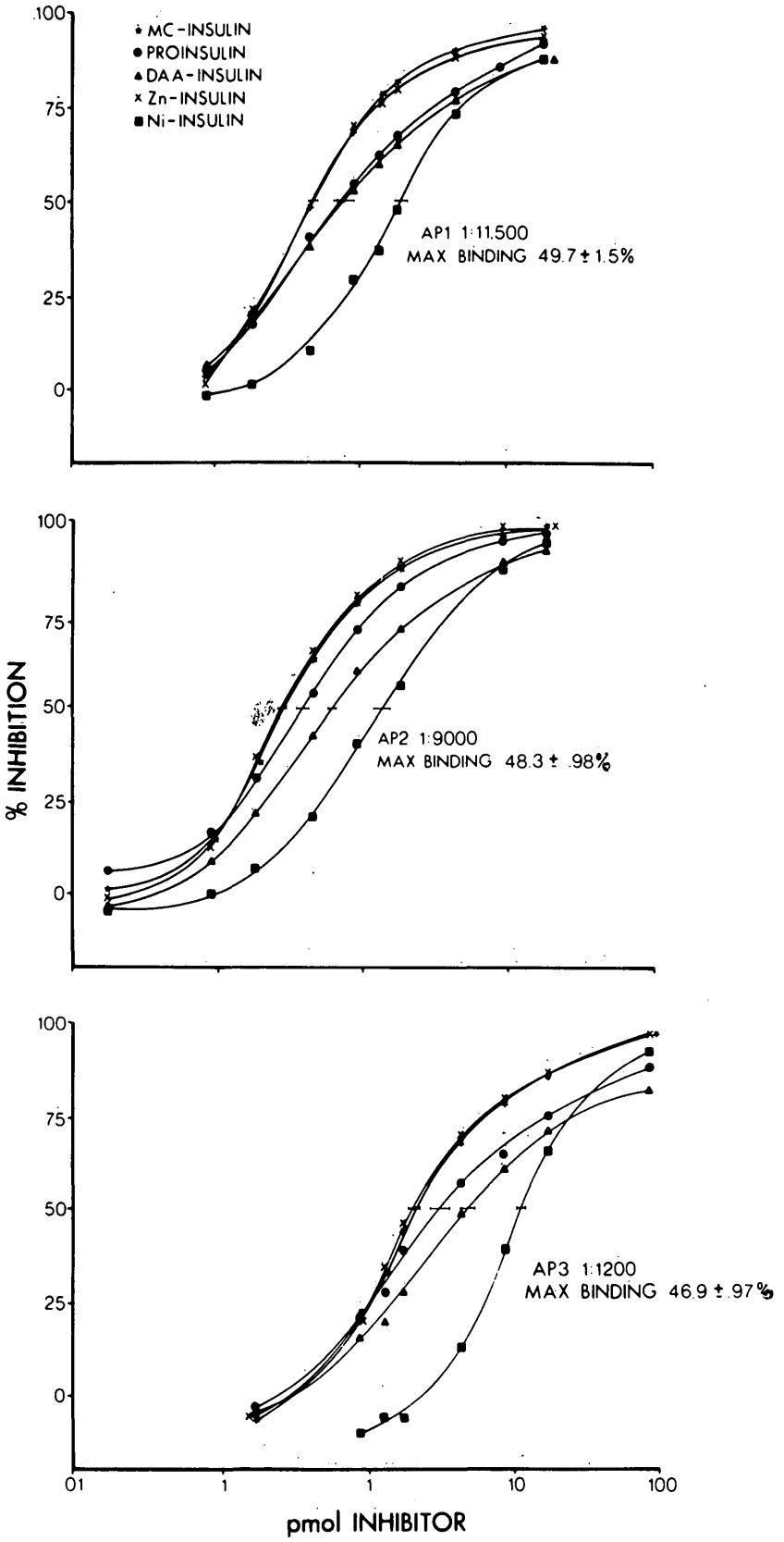


FIGURE 1. Radioimmune inhibition assay (RIIA). Percent inhibition vs. picomoles of inhibitor per tube for each insulin antiserum pool (AP). Each point represents a mean of at least three determinations. The $\bar{x}$ ± SEM maximum binding obtained with the dilution of each AP in the absence of any inhibitor was determined from at least three experiments. The inhibitors were monocomponent insulin (MC-insulin), proinsulin, desalanine-desasparagine insulin (DAA-insulin), zinc-insulin (Zn-insulin), and nickel-insulin (Ni-insulin).

peak hyperglycemia was at 120 min followed by a more sustained hyperglycemia at 3 and 4 h. The antiserum pool with the low radioimmune titer (AP3) caused a hyperglycemia that peaked significantly lower at 60 min and was back to normal limits at 180 min, resulting in a qualitatively attenuated diabetogenic activity.

The diabetogenic activity was calculated from the differences in area between the respective hyperglycemia curves

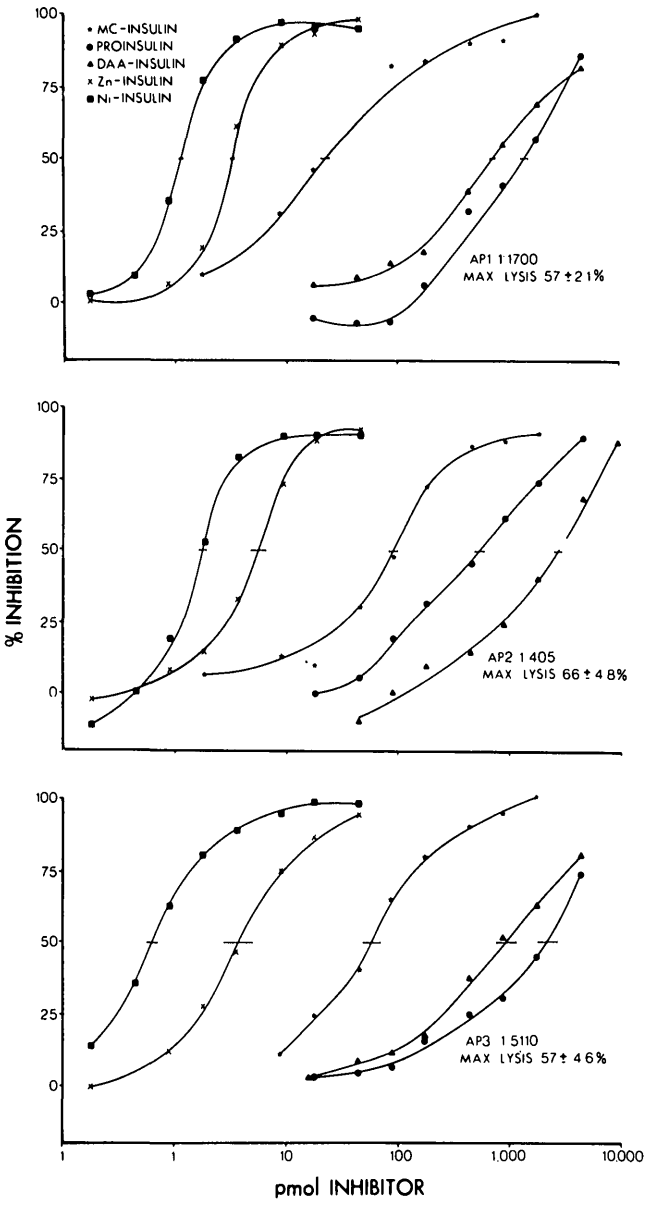


FIGURE 2. Immune hemolysis inhibition assay (IHIA). Percent inhibition vs. picomoles of inhibitor per tube for each insulin antiserum pool (AP). Each point represents at least three determinations. The $\bar{x}$ ± SEM maximum lysis obtained with the dilution of each AP in the absence of any inhibitor was determined from at least three experiments. The inhibitors were monocomponent insulin (MC-insulin), proinsulin, desalanine-desasparagine insulin (DAA-insulin), zinc-insulin (Zn-insulin), and nickel-insulin (Ni-insulin).

of each insulin antiserum pool for each of four doses and the curve obtained with normal guinea pig serum. The diabetogenic activity was linearly related to the 0.05- and 0.1-ml doses for all three pools of insulin antisera. A maximal diabetogenic activity was calculated at the 0.2-ml dose for AP1 and AP2, but it was linearly related to the 0.05-ml through 0.4-ml doses for AP3. The relative diabetogenic activity measured for each of the three antiserum pools (AP1 > AP2 > AP3) was maintained at each dose (Figure 5).

A maximum hyperglycemia (420 mg/dl) was obtained when 0.1 ml each of AP1 and AP2 were injected. The diabetogenic activity observed with higher doses (Figure 5) was caused by a sustained rather than an elevated hyperglycemia. The maximum hyperglycemia (380 mg/dl) induced with AP3 was observed only when 0.4 ml of antiserum was injected. Therefore, the increase of diabetogenic activity at higher doses for AP3 (Figure 5) reflected both an elevated and a sustained hyperglycemia.

TABLE 2
Radioimmune inhibition assay (RIIA)
[picomoles of inhibitor required per tube for 50% inhibition of each antiserum pool (AP)]

Inhibitor	Antiserum Pool AP-1	AP-2	AP-3
MC-insulin	0.45 ± 0.02	0.28 ± 0.02	2.19 ± 0.10
Proinsulin	0.71 ± 0.04	0.37 ± 0.03	3.28 ± 0.52
DAA-insulin	0.77 ± 0.12	0.59 ± 0.03	4.82 ± 0.49
Zn-insulin	0.47 ± 0.04	0.28 ± 0.02	1.98 ± 0.04
Ni-insulin	1.88 ± 0.16	1.31 ± 0.16	11.60 ± 0.77

Results are presented as mean values ± SEM for at least three determinations.

A strong positive correlation ($r = 0.9868$, $P < 0.01$) between diabetogenic activity and radioimmune titer was observed (Figure 6). Figure 6 was generated from doses in the linear portion of the dose response curves for each AP (Figure 5; 0.05- and 0.1-ml doses). Although the correlation remained significant ($P < 0.01$) when higher doses were included in the calculation, both the slope and the correlation decreased (Table 4). This is best explained by the fact that, for high doses of AP1 and AP2 (0.2 and 0.4 ml), the blood glucose remained elevated at 4 h, when the experiment was terminated. Had the experiment been permitted to continue until glycemia returned to normal limits, it is likely that the diabetogenic activity vs. radioimmune titer plot would have had similar correlations and slopes when the higher doses were included in the calculations.

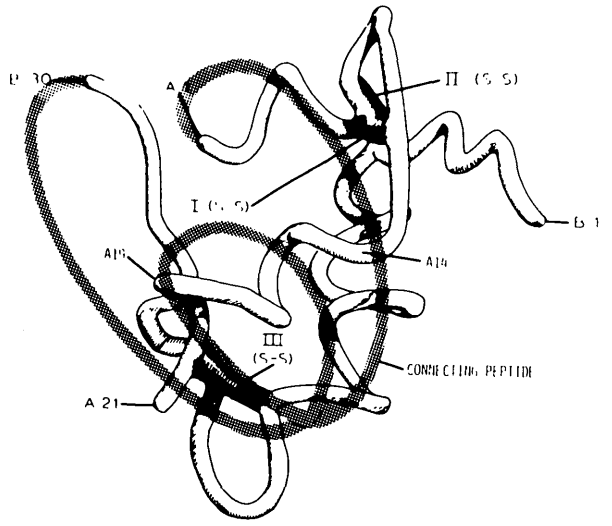
A negative correlation ($r = 0.8338$, $P < 0.01$) between diabetogenic activity and immune hemolysis was observed. This negative correlation was expected, since the antisera were selected to have inversely related radioimmune and immune hemolysis titers. In preliminary experiments, where the diabetogenic activity of insulin antisera was determined randomly, a positive correlation between diabetogenic activity and radioimmune titer and no correlation between diabetogenic activity and immune hemolysis titer were observed.

These observations are submitted as evidence that insulin in vivo—in the circulation, in the cell surface receptor, or

TABLE 3
Immune hemolysis inhibition assay (IHIA)
[picomoles of inhibitor required per tube for 50% inhibition of each antiserum pool (AP)]

Inhibitor	Antiserum Pool AP-1	AP-2	AP-3
MC-insulin	22.10 ± 1.62	86.50 ± 9.22	56.20 ± 11.70
Proinsulin	1330.00 ± 76 70	528.00 ± 57.90	2150.00 ± 475.00
DAA-insulin	719.00 ± 47.50	2610.00 ± 174.00	916.00 ± 162.00
Zn-insulin	3.31 ± 0.10	5.22 ± 0.87	3.65 ± 1.04
Ni-insulin	1.17 ± 0.05	1.67 ± 0.12	0.64 ± 0.07

Results are presented as mean values ± SEM for at least three determinations.



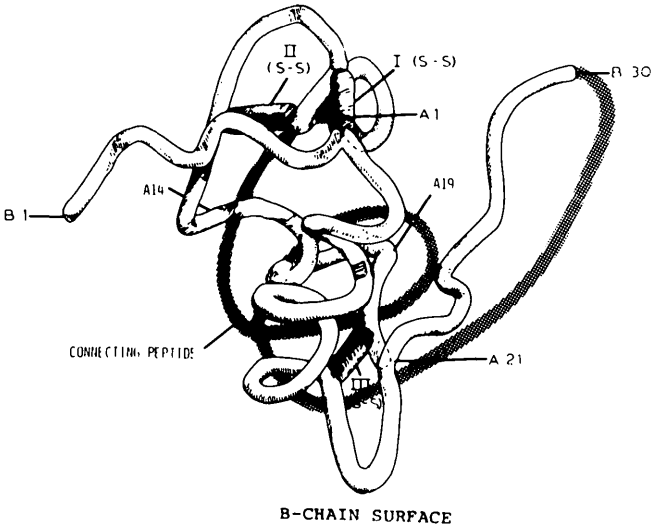


FIGURE 3. Views of the A- and B-chain surfaces of a worm model of insulin. The models were constructed from the alpha carbon coordinates (graciously supplied by Guy Dodson and Dorothy Hodgkin) of the 51 amino acid residues in insulin. The projection of the connecting peptide on the solvent surface is proposed from the data presented in the text. I (S-S) is the interchain disulfide bridge (A-6 to A-11). II (S-S) is the interchain disulfide bridge (A-7 to B-7). III (S-S) is the interchain disulfide bridge (A-20 to B-19). A-1 is the position of the alpha carbon of the N terminal Gly of the A-chain. B-1 is the position of the alpha carbon of the N terminal Phe of the B-chain. A-21 is the position of the alpha carbon of the C terminal Asp of the A-chain. A-14 and A-19 are the positions of the alpha carbons of the A-chain Tyr to which >90% of the iodine is substituted in the ^{125}I-monoiodotyrosylinsulin used in these experiments.

both—is accessible for interaction with insulin antibodies directed to determinants on the B-chain surface. These interactions may be of higher affinity or more accessible than in vivo interactions with insulin antibodies directed to determinants on the A-chain surface.

TABLE 4
Calculation of diabetogenic activity vs. radioimmune titer

Doses (ml) included	Correlation (R)	Significance (P)	Slope
0.05, 0.1	+0.9868	0.01	28
0.05, 0.1, 0.2	+0.9057	0.01	26
0.05, 0.1, 0.2, 0.4	+0.7480	0.001	21

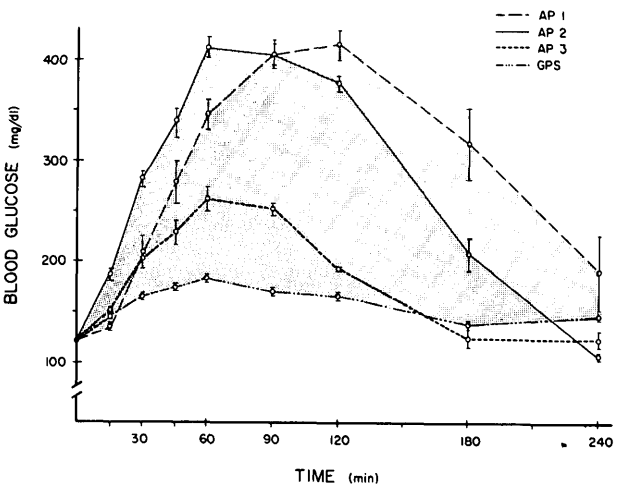


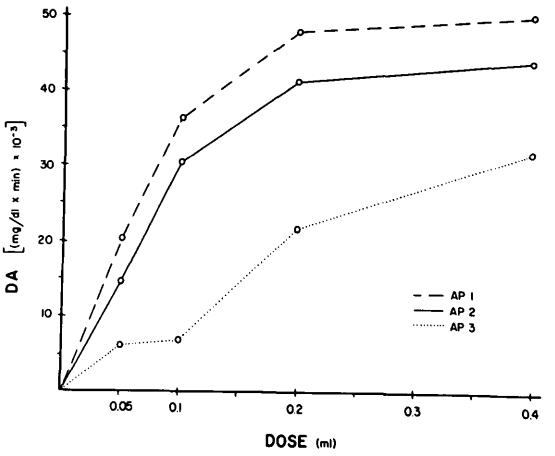
FIGURE 4. Blood glucose ($\bar{x}$ ± SEM) vs. time after intraperitoneal injection of 0.1 ml of each insulin antiserum pool (AP) or normal guinea pig serum. N = 10 for each point on each AP curve through 120 min. N = 6 for the 180-min and 240-min points. The normal guinea pig serum curve is a composite of the four doses 0.05, 0.1, 0.2, and 0.4 (N = 20), since each dose elicited the same response. The area under this normal guinea pig serum curve was subtracted from the area under each AP curve for the determination of diabetogenic activity for each dose of each AP (Figure 5).

DISCUSSION

In preliminary studies, a positive correlation between the level of AB_R in insulin antisera and the level of hyperglycemia induced was observed when the antisera were injected. In these same experiments, there was no correlation between the level of AB_H in the insulin antiserum and the level of hyperglycemia induced.

In the experiments described in this manuscript, we successfully predicted the hyperglycemia-inducing potency of three antiserum pools. We expected the two antiserum pools—AP1 and AP2—comprised of antisera with elevated levels of AB_R and low levels of AB_H to be significantly more potent inducers of hyperglycemia than the antiserum

FIGURE 5. Diabetogenic activity vs. dose for each insulin antiserum pool (AP). The diabetogenic activity (mg glucose/dl × min) was calculated from the difference in an area under the curve of hyperglycemia vs. time for each AP dose and the glycemia curve obtained with normal guinea pig serum (Figure 4).



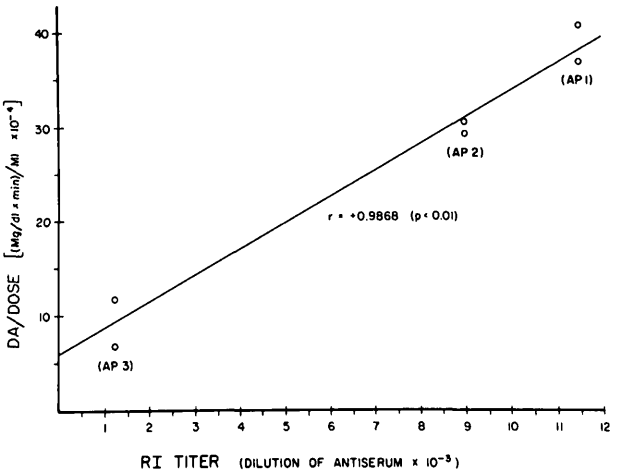


FIGURE 6. Diabetogenic activity (0.05- and 0.1-ml doses) vs. radioimmune titer for each antiserum pool (AP). The slope of this line of best fit is an estimate of the diabetogenic activity (see Figure 5) that can be expected when low doses (<0.1 ml) of insulin antiserum containing radioimmune antibody titers in the designated range are injected intraperitoneally into mice.

pool—AP3—comprised of antisera with low levels of AB_R and elevated levels of AB_H.

The positive correlation between radioimmune titer and diabetogenic activity in contrast with the lack of (or negative) correlation between immune hemolysis titer and diabetogenic activity is evidence that AB_R are more potent inducers of hyperglycemia than are AB_H. This supports the probability that insulin in vivo, either in circulation, on the cell surface, or both, is oriented to permit interaction with AB_R to a greater degree than is AB_H. There are at least three possible explanations for this phenomenon. First, AB_H may be of relatively low affinity compared with AB_R and, thus, less effective in causing hyperglycemia. Second, AB_H may be directed toward determinants on insulin, which are relatively less accessible in vivo than those to which AB_R are directed. Third, AB_H may be directed toward a site not essential for insulin's activity while AB_R may be directed toward an active site. None of these possibilities excludes the other nor do any of them separately or collectively negate the early interpretations[4-6] that the hyperglycemia induced by the injection of guinea pig insulin antibodies is a result of the interaction between antibodies and endogenous insulin in the circulation.

The difference in the two assays is best attributed to the reaction of antibody with the insulin indicator in each assay —^{125}I-monoiodoinsulin in the RIIA and sheep red blood cells coated with Zn-insulin aggregates in the IHIA. There are at least three differences in the indicator insulins. First, the insulin in the radioimmune system is soluble, while that in the immune hemolysis system is attached to an insoluble matrix. This may affect the affinity required for, and the kinetics of, the antibody-indicator interaction. Second, the Zn-insulin linked to red cells in the immune hemolysis system is at a concentration (1 mg/ml) that exists primarily in hexamers.[8] When these Zn-insulin hexamers are linked to red cells with bisdiazobenzadine, cross linking within the hexamer may occur, resulting in an indicator in which certain determinants are buried and unavailable for antibody binding.[2,8] Such determinants on the ^{125}I-monoiodoinsulin indicator in the radioimmune system would be accessible for antibody interaction. Third, the number of insulin molecules available for antibody interaction is four orders of magnitude greater in the immune hemolysis system than in the radioimmune system.

The immunologic reactivity of proinsulin with AB_R was 15-fold greater than that with AB_H (see Figures 1 and 2 and Tables 2 and 3). Therefore, AB_R are directed toward sites on the insulin molecule that are less perturbed on proinsulin than the sites to which AB_H are directed. Similar observations with other antisera have been previously reported.[2] It has been proposed[15,16] that the connecting peptide in proinsulin is in apposition to surface residues of the A-chain of the insulin molecule (Figure 3). It follows that, in proinsulin, the determinants least accessible for antibody interaction are on the A-chain surface. This logic supports the postulate that AB_H are directed toward A-chain surface determinants and AB_R are directed toward B-chain surface determinants (Figure 3).

Extending this logic, it is proposed that, on Ni-insulin, the poorest inhibitor of AB_R but the most effective inhibitor of AB_H, the B-chain determinants are severely perturbed. The A-chain determinants, on the other hand, are stabilized and immunologically more reactive than the other derivatives tested when each is compared with MC-insulin. The B-chain determinants on Zn-insulin are unperturbed and the A-chain determinants are markedly more stable than on MC-insulin. Both the B-chain and A-chain determinants are perturbed in DAA-insulin, although the A-chain determinants are more severely perturbed. These observations are submitted as additional evidence to the previous reports[2,4] that AB_R are directed to B-chain surface determinants and AB_H to A-chain surface determinants.

Since AB_R are more potent inducers of hyperglycemia than AB_H and because they are directed toward the B-chain determinants of insulin, it is proposed that antibody binding to the B-chain determinants of insulin is either of higher affinity, more readily accessible in vivo, or that this surface of insulin is needed to express its biologic activity. None of these possibilities excludes the other.

ACKNOWLEDGMENTS

This work was supported by grants from the United States Public Health Service (AM-13441-11) and from the Kroc Foundation.

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