Structural Studies of Insulin and Insulin Derivatives Using Various Immunologic Indicators and Antibody Populations

Edward R. Arquilla, M.D., Ph.D., Raymond J. Dorio, M.D., and Thomas M. Brugman, B.S., Irvine, California

SUMMARY

These studies suggest that the immunologic indicator in the radioimmune assay, ^{125}I-iodoinsulin, selects antibody populations from within the antiserum that interact with determinants distant from the solvent surface on the insulin molecule to which iodine is substituted. Evidence is presented that the connecting peptide of proinsulin is in close proximity to regions on the solvent surface of the A-chain of insulin that include the tyrosyl residues at A-14 and A-19.

A marked immunologic cross-reaction between derivatives of insulin with perturbations in the regions of tyrosyl A-14 and A-19 was noted in the radioimmune assay employing desalanine-(B-30)-desasparagine-(A-21)-insulin antiserum. This observation is consistent with the presence of a restricted population of antibodies in such antisera that is directed toward immunologic determinants in or near the insulin dimer site.

The apparent immunologic activity of insulin derivatives depends on which antibody populations from the antiserum pool can react with the immunologic indicator employed on the one hand and on the composition of antibodies in that antiserum on the other. These studies indicate that the specificity of antibody populations in a given antiserum can be identified and their levels quantitated with several assay systems, each employing one of a variety of indicators. DIABETES *25*:397-403, May, 1976.

The insulin antiserum used to measure insulin in immunoassay systems is composed of several populations of antibodies. Each antibody population is in turn directed toward one of several immunologic determinants on the insulin molecule. Previous observations from this laboratory demonstrated that several insulin derivatives exhibited higher relative immunologic activity in a commercial radioimmune assay (RIA) than in an immune hemolysis-inhibition assay (IHIA).[1] It was not clear whether these differences in immunologic activity were due to differences in the two antiserum pools employed or to other parameters, such as differences in structure of the two immunologic indicators, ^{125}I-iodoinsulin in the RIA and the insulin-coated sheep red cells in the IHIA. In order to resolve this question a single antiserum pool was used in both assay systems.

From the results obtained and the knowledge of the structure of insulin in two Zn rhombohedral crystals as determined by x-ray crystallography[2] it is possible to propose that certain immunologic determinants on the insulin molecule are more directly involved in the RIA than in the IHIA. In addition, evidence is presented that the connecting peptide of proinsulin is in close proximity to the tyrosyl A-14 and A-19 and therefore covers the solvent-exposed portion of the A-chain of insulin.

MATERIALS AND METHODS

Insulin and Insulin Derivatives

Bovine insulin, 10 times recrystallized, was a gift from the Novo Corporation. Two derivatives with perturbations of the A-chain tyr (A-14 and A-19) were prepared. A preparation of 3-mononitrotyrosyl-insulin was made according to the method of Morris, Mercola, and Arquilla.[3] This was a homogeneous preparation of 3-mononitrotyrosyl-insulin containing approximately 76 per cent of the insulin molecules, with the nitro group substituted on tyr A-14, and 14 per cent of the molecules with the nitro group, substituted on tyr A-19. The remaining 10 per cent of the insulin molecules contain nitro groups substituted on the two B-chain tyr (B-16 and B-26). The other derivative with perturbations of A-14 and A-19 was ^{127}I-monoiodotyrosyl-insulin. It was prepared accord-

A preliminary report of part of these data was presented in Atlantic City, at the Annual Meeting of the Federation of American Societies for Experimental Biology. Fed. Proc. *32*:312, 1973.

From the Department of Pathology, University of California, Irvine, California 92717.

Address reprint requests to Edward R. Arquilla, M.D., Department of Pathology, University of California, Irvine 92717.

Accepted for publication January 13, 1976.

ing to a modification of the lactoperoxidase method[4] as previously described.[5] This preparation of ^{127}I-monoiodotyrosyl-insulin has been demonstrated to have approximately 76 per cent of the insulin molecules with iodine substituted on tyrosine A-14 and about 19 per cent of the insulin molecules substituted on A-19. The remaining 5 per cent of the insulin molecules contain iodine substituted on B-chain residues. Desoctapeptide-(B-23 to B-30)-insulin was prepared as described by Bromer and Chance.[6] Desalanine-(B-30)-desasparagine-(A-21)-insulin was prepared by the exhaustive digestion of insulin with carboxypeptidase A as previously described by Slobin and Carpenter[7] and purified by a column chromatography method previously described by Brugman and Arquilla.[8]

All insulin derivatives were prepared from bovine insulin obtained from Elanco Inc., Indianapolis. All derivatives migrated as single components in 7 per cent acrylamide gel electrophoresis, pH 8.6, previously described.[8] This procedure can detect a 2 per cent contamination. Bovine proinsulin was a gift from Dr. Donald F. Steiner. Guinea pig insulin was a gift from Dr. Cecil C. Yip. The commercial ^{125}I-iodoinsulin used as an immunologic indicator in the RIA was obtained from the Amersham Searle Corporation. It contained approximately 50 mCi. per mg. The carrier-free ^{125}I-monoiodotyrosyl insulin also used as an immunologic indicator in the RIA was prepared according to the method of Sodoyez et al.[9] This preparation contained approximately 250 mCi. per mg.

The concentrations of insulin and insulin derivatives used in these experiments were determined spectrophotometrically in a 0.01-N HCl solution with a Cary M-14 recording spectrophotometer according to the A_{276}nm values in table 1.

TABLE 1

Molar absorbtivity for insulin and insulin derivatives*

Insulin	5,950
^{127}I-Monoiodotyrosyl-insulin	6,310†
3-Mononitrotyrosyl-insulin	10,120
(DOP)‡	4,600
(DAA)‡	5,950
Proinsulin	5,950
Guinea pig insulin	4,600

*These molar absorbtivity (A_{276}nm) values of insulin and insulin derivatives in an acid solvent (0.01 N HCl) were calculated by assuming that each nonionized tyrosine contributes 1,350 to the molar absorbtivity of insulin. DOP and guinea pig insulin only have three tyrosyl residues per molecule. It was assumed that 3-nitrotyrosine contributes 5,520 to the molar absorbtivity at 276 nm.[3] Proinsulin was assumed to have the same A_{276}nm value as insulin since the C peptide contains no histadyl or tyrosyl residues.

†According to Sodoyez, J. C., et al.[9]

‡See figure 1 and text.

Antisera

The single antiserum pool used in both assay systems was a pool from at least 10 mongrel guinea pigs immunized with 10 × recrystallized bovine Zn insulin as previously described.[10,11] The des-Ala-des-Asp-insulin antiserum pool used in these experiments was identical to that used by Brugman and Arquilla.[8]

The titers of each of the individual insulin and des-Ala-des-Asp-insulin antisera collected from each of the guinea pigs and titers of the final antiserum pools were determined by the passive immune hemolysis reaction. This method for measuring insulin antibodies relies on the interaction of antibodies with antigen-coated red cells in the presence of excess amounts of complement. It has proved to be an accurate measure of the relative antibody concentration.[10-12]

Immune Hemolysis Inhibition Assay (IHIA)

The IHIA was used as one of the systems to measure the immunologic cross-reactivity of the various insulin derivatives tested. This assay system employs 8.7 × 10^6 red cells coated with insulin, sufficient antibodies to lyse 80 per cent of the red cells, three units of complement and variable amounts of inhibitor. In the presence of an inhibiting material a portion of the antibodies in the reaction will complex with the inhibitor, resulting in a decrease in the number of antibodies free to react with the red cell conjugates. Immune hemolysis inhibition is quantitated by measuring the decrease in the amount of hemoglobin released as more inhibitor is added to the system. The degree of cross-reaction is expressed by the ratio of moles of the homologous antigen (insulin) to the moles of the cross-reacting antigen necessary for 50 per cent inhibition, figure 1.

Insulin antiserum (0.1 ml.) at a dilution of 1/220, 0.1 ml. insulin-coated red cells, and 0.1 ml. inhibitor were incubated one hour in a New Brunswick gyratory water-bath shaker at 4° C., following which three units of guinea pig complement (0.1 ml.) were added to each tube and the mixture further incubated for 30 minutes at 37° C. The reaction was stopped with 1 ml. cold-phosphate-buffered saline, pH 7.4, and the unlysed cells pelleted by centrifugation at 2,000 g. The supernatants were decanted and the per cent lysis was calculated from the amount of hemoglobin released, which was measured spectrophotometrically at 414 nm.

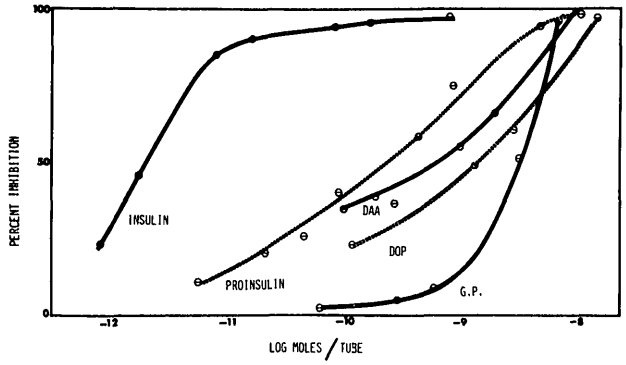

FIG. 1. Immunologic activities of insulin and insulin derivatives by immune hemolysis-inhibition assay. The immune hemolysis-inhibition assay (IHIA) was carried out as described in the text. The per cent cross-reaction is calculated from the ratio of moles of insulin to moles of proinsulin, desalanine-(B-30)-desasparagine-(A-21)-insulin (DAA), desoctapeptide-(B-23 to B-30)-insulin (DOP), or guinea pig insulin (G.P.) needed for 50 per cent inhibition. Bovine insulin was assumed to be 100 per cent active.

Radioimmune Assay (RIA)

The radioimmune assay was performed with a modification of the charcoal method for separating bound and free insulin developed by Herbert et al.[13] All dilutions were made in Na barbital-acetate buffer with 0.855 per cent NaCl and 350 mg./100 ml. human serum albumin (Merck Sharpe and Dohme). The dextran-coated charcoal solution was prepared by mixing 0.5 per cent solution of dextran T70 (Pharmacia) volume per volume with a 5 per cent solution of Norit A decolorizing carbon (Amend Drug and Chemical).

Insulin antiserum or des-Ala-des-Asp-insulin antiserum was serially diluted and incubated with the commercial ^{125}I-iodoinsulin or carrier-free ^{125}I-monoiodotyrosyl-insulin for 40 hours at 37° C. in a total volume of 0.5 ml. Equilibrium was attained after 24 hours, and no loss of binding was detected up to 72 hours. The free insulin was removed by adsorption to dextran-coated charcoal (2 ml.) and centrifuged for 30 minutes at 2,000 g. The radioactivity of the antibody-bound insulin in the supernates was measured in a Packard Autogamma counter. The dilution of antiserum that bound 80-90 per cent of maximum binding was used in the RIA. Maximum binding of the commercial ^{125}I-iodoinsulin with excess antiserum was approximately 75 per cent of the total c.p.m. and 100 per cent when the carrier-free ^{125}I-monoiodotyrosylinsulin was used.

The incubation mixture for the RIA consisted of 0.1 ml. of the predetermined antiserum dilution, 0.1 ml. of a known amount of inhibitor (insulin or insulin derivative), and 0.1 ml. radioactive indicator. The ratio (C_o/C_i) of the number of counts remaining in the supernate (bound to antibody) in the absence of any inhibiting material to the number of counts in the presence of inhibitor was plotted against the concentration of inhibitor. The curves were generated by paired data analysis. When C_o/C_i equals 2.0, half of the radioactive indicator has been displaced from the antibody by the inhibitor. The relative immunologic activities of insulin and various insulin derivatives were calculated at that point, figure 2.

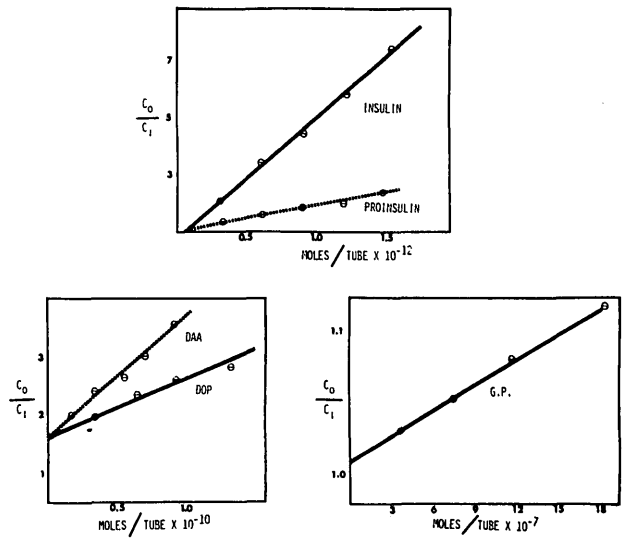

FIG. 2. Immunologic activities of insulin and insulin derivatives by radioimmune assay. The radioimmune assay (RIA) was carried out as described in the text. C_o represents the number of counts in the supernate in the absence of insulin or any inhibiting material. C_i represents the radioactivity in the supernate in the presence of insulin or insulin derivative. The immunologic activity was calculated by determining the moles of des-alanine-(B-30)-desasparagine-(A-21)-insulin (DAA), desoctapeptide-(B-23 to B-30)-insulin (DOP), or proinsulin to the moles of insulin needed for a ratio $C_o/C_i = 2$. In the case of guinea pig insulin (G.P.) the ratio $C_o/C_i = 2$ was extrapolated. Bovine insulin was assumed to be 100 per cent active.

RESULTS

The Immunologic Activity of Insulin Derivatives in the Immune-Hemolysis Inhibition Assay (IHIA) and in the Radioimmune Assay (RIA)

The immunologic activities of the various insulins and insulin derivatives were measured in the two assay systems with a single antiserum pool, table 2. In each of these assay systems bovine insulin was considered to have 100 per cent immunologic activity.

The relationship between the level of antibody present and the amount of insulin needed to inhibit that level of antibody was of the same order of mag-

TABLE 2

Per cent immunologic activity of insulin and insulin derivatives in the radioimmune assay (RIA) and in the immune hemolysis-inhibition assay (IHIA)*

	RIA†	IHIA‡
Insulin	100	100
Proinsulin	22.2 ± 5.1	0.78 ± 0.31
(DAA)§	0.68 ± 0.23	0.15 ± 0.084
(DOP)§	0.32 ± 0.093	0.069 ± 0.043
Guinea pig insulin	0.0037 ± 0.0047	0.047 ± 0.030

*The same insulin antiserum was used in both the RIA and IHIA. Values expressed are means ± standard deviation with insulin representing 100 per cent immunologic activity. All immunologic activities in the RIA are significantly different from those observed in the IHIA, $P < 0.01$.

†The commercial ^{125}I-iodoinsulin indicator was obtained from the Amersham Searle Corporation. Immunologic activities of insulin and each of the insulin derivatives were determined as explained in figure 2.

‡The immunologic activities of insulin and each of the insulin derivatives were determined as explained in figure 1.

§See figure 1 and text.

nitude in both assays. In the IHIA, 50 per cent inhibition of lysis occurred in the presence of approximately 1.5×10^{-12} M of insulin with a 1/220 antiserum dilution. In the RIA 50 per cent displacement of the commercial ^{125}I-iodoinsulin indicator (C_o/C_i = 2) occurred in the presence of 0.35×10^{-12} M of insulin with a 1/640 antiserum dilution, figures 1 and 2.

The relative immunologic activity of proinsulin was approximately 25 times greater in the RIA than in the IHIA, 22.2 per cent and 0.78 per cent, respectively. These results are consistent with previous results from experiments using a different antiserum in each of the two assay systems.[1] Guinea pig insulin had less relative immunologic activity in the RIA than in the IHIA, 0.0037 per cent and 0.047 per cent, respectively. Desoctapeptide-insulin and des-Ala-des-Asp-insulin both had greater relative immunologic activity in the RIA than in the IHIA.

All of these substances inhibited the immune hemolysis reaction completely when used in excess, figure 1. The same materials exhibited an immunologic activity in the RIA that gave linear dose-response curves. The correlation coefficient (r) of the dose response for insulin, proinsulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin was always >0.95. In spite of the very attenuated immunologic activity of guinea pig insulin as expressed by the flat slope, a significant dose-response curve (r) (0.78) was consistently obtained, figure 2.

The commercial ^{125}I-iodoinsulin employed contains a mixture of unreacted insulin and iodinated insulins. Since immunologic indicators can vary, it was important to test whether the derivatives that were used reacted in a similar fashion when a homogeneous preparation of carrier-free ^{125}I-monoiodotyrosyl-insulin was the immunologic indicator. Experiments were designed in which the commercial ^{125}I-iodoinsulin and the carrier-free ^{125}I-monoiodotyrosyl-insulin were tested in parallel. Equal molar amounts of all indicators, identical dilutions of antiserum, and comparable concentrations of inhibitor (proinsulin) were used in the two parallel assay systems. The cross-reaction observed between proinsulin and insulin with commercial ^{125}I-iodoinsulin as the indicator was 17.7 per cent ± 1.2, whereas the cross-reaction between proinsulin and insulin with carrier-free ^{125}I-monoiodotyrosyl-insulin as the indicator was 21.2 per cent ± 1.6 per cent. In these experiments comparable levels of insulin were required to inhibit 50 per cent of the antibodies capable of binding the respective indicators. These results lend reliability to the interpretation that the immunologic indicator in the insulin RIA selects antibodies that are operative against determinants distant from the solvent surface of the insulin molecule. They further support the conclusion that the connecting peptide in proinsulin closely approximates the solvent surface of the A chain.

Radioimmune Assay with Des-Ala-Des-Asp-Insulin Antiserum

The foregoing experiments demonstrate that the ability to measure the immunologic activity of insulin and various insulin derivatives with an antiserum is dependent upon the nature of the indicator used in the assay system. Since the influence of antisera with restricted populations of antibodies could also influence the RIA, it was provocative to study des-Ala-des-Asp-insulin antiserum in the RIA. Des-Ala-des-Asp-insulin antisera contain populations of antibodies directed toward a restricted portion of the insulin molecule.[8] The system employed in these experiments comprised des-Ala-des-Asp-insulin antiserum, commercial ^{125}I-iodoinsulin indicator, and various insulin derivatives as inhibitors.

A very significant cross-reaction between insulin (77 per cent), ^{127}I-monoiodotyrosyl-insulin (80.1 per cent), 3-mononitrotyrosyl-insulin (57.8 per cent), and proinsulin (19.4 per cent) with des-Ala-des-Asp-insulin antiserum was noted, table 3. Conversely, des-Ala-des-Asp-insulin cross-reacts poorly (0.68 per cent) with insulin antiserum, table 2.

TABLE 3

Cross-reaction of insulin and insulin derivatives in the radioimmune assay using desalanine-(B-30)-desasparagine-(A-21)-insulin antisera*

Insulin or derivative	% Activity
DAA†	100
Insulin	77.0 ± 5.0
DOP†	29.6 ± 1.2
Iodoinsulin	80.1 ± 9.0
Nitroinsulin	57.8 ± 10.4
Proinsulin	19.4 ± 3.8

*A radioimmune assay was constructed using the same desalanine-desasparagine antisera as used in the IHIA by Brugman and Arquilla.[8] Values expressed are means ± standard deviation. Activity was determined as explained in figure 2. The homologous antigen des-Ala-des-Asp-insulin (DAA) was assumed to have 100 per cent immunologic activity.

†See figure 1 and text.

The results support the previous observations[8] that the antibody populations in des-Ala-des-Asp-insulin antiserum that cross-react with insulin are directed toward portions of the insulin molecule distant from the solvent surface that contains the A-14 and A-19 tyrosyl residues and is also covered by the connecting peptide in proinsulin. The restricted population of antibodies probably directed toward determinants in the dimer site is able to cross-react to a significant degree with insulin and also 3-mononitrotyrosyl-insulin and ^{127}I-monoiodotyrosyl-insulin, each of which contains perturbations of the A-chain tyrosines. The A-chain perturbations induced by the nitro group and iodine substitution in the respective derivatives did not appreciably affect the cross-reaction with the des-Ala-des-Asp antibodies. Consequently, the populations of antibodies and the determinants on insulin with which they react significantly influence the immunologic activity of derivatives or species of insulins tested.

DISCUSSION

Comparison of Immunologic Activity of Insulin Derivatives in the Immune Hemolysis Inhibition Assay (IHIA) to That in the Radioimmune Assay (RIA)

Previous studies[1] suggested that antibody populations operative in the RIA are directed toward determinants distant from the solvent surface of the insulin molecule, figure 3. This surface contains the A-14 and A-19 tyrosyl residues, which are iodinated to a variable degree in ^{125}I-iodotyrosyl-insulin. It has been suggested that this solvent surface is also the portion of the molecule that is covered by the connecting peptide in proinsulin, figure 3.[14]

In the previous studies the antiserum employed in the IHIA was prepared in our laboratory and was different from the antiserum used in the commercial RIA. Furthermore, the concentration of insulin, insulin derivatives used as inhibitors, and level of antibodies in the RIA were significantly less than those used in the IHIA. In the current experiments, precautions were taken to use the identical antiserum pool in both the RIA and the IHIA. The relationship between the level of antibody present and the amount of insulin needed to inhibit that level of antibody was of the same order of magnitude in both the RIA and the IHIA and lends confidence to the reliability of the results.

The increased immunologic activity of proinsulin and, conversely, the decreased activity of guinea pig insulin in the RIA is best explained by the nature of the immunologic indicators, commercial ^{125}I-

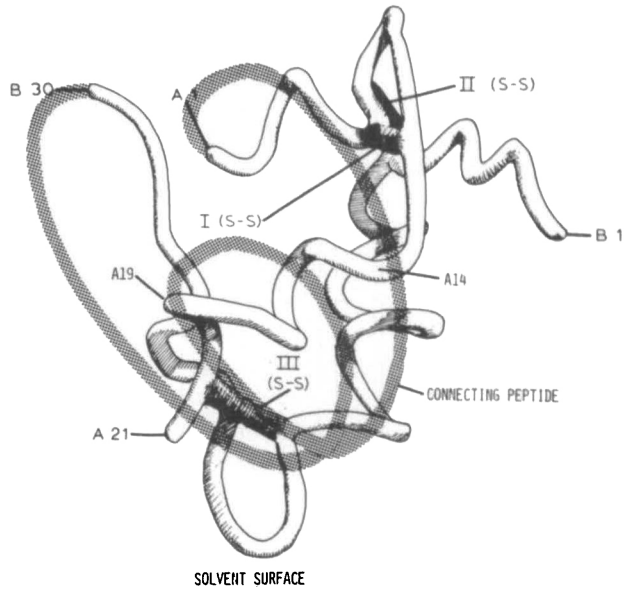

FIG. 3. A view of the solvent surface of a worm model of insulin. The model was constructed from the alpha carbon coordinates (graciously supplied by Prof. Dorothy Hodgkin) of the 51 amino acid residues in insulin. The projection of the connecting peptide on the solvent surface is a speculation from 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. B-30 is the position of the alpha carbon of the C terminal Ala of the B-chain. A-14 is the position of the alpha carbon of the A-chain tyr to which approximately 76 per cent of iodine is substituted in the ^{127}I-monoiodotyrosyl-insulin used in these experiments. A-19 is the position of the alpha carbon of A-chain tyr to which approximately 14 per cent of iodine is substituted in the ^{127}I-monoiodotyrosyl-insulin used in these experiments.

iodoinsulin, and insulin-coated red cells employed in the two assays. The immunologic indicator in the RIA was commercial ^{125}I-iodoinsulin that contained a mixture of unreacted insulin and iodinated insulin, having a specific activity of 50 mCi. per mg. A homogeneous carrier-free ^{125}I-monoiodotyrosyl-insulin preparation theoretically should contain approximately 350 mCi. per mg. The precise location of the substitution in the commercial preparation is unknown; however, it is likely that the majority of the iodines are attached to the A-chain tyrosines.[17] In view of the heterogeneity of commercial indicator we felt it was necessary to substantiate the cross-reaction between insulin and a derivative (proinsulin) with an iodoinsulin indicator of known homogeneity. The experiments with the carrier-free ^{125}I-monoiodotyrosyl-insulin as the indicator support the contention that insulin indicators labeled with iodine primarily in the A-chain do indeed select antibodies from within the antiserum pool that are directed toward sites other than the regions in proximity to the A-14 and A-19 tyrosyl residues on the solvent surface of the insulin molecule, figure 3. It is postulated that antibodies directed toward determinants near the A-14, A-19 tyrosyl regions combine poorly with the iodoinsulin indicator used in the RIA because of the steric hindrance caused by the substituted iodine.

The indicator in the IHIA is insulin-bound to sheep red cells with bisdiazobenzidine (BDB).[10,11] Studies with albumin have demonstrated that BDB interacts with several functional groups on the albumin molecule.[15,16] It is reasonable to assume that BDB interacts in a similar fashion with the insulin molecule, and, therefore, several faces of the insulin molecule on the red cells are probably exposed for antibody interaction.

Derivatives with perturbations on the solvent surface in the region of A-14 and A-19 would have a greater apparent immunologic activity in the RIA than the IHIA. It therefore follows that the significantly greater immunologic activity of proinsulin in the RIA than in the IHIA is evidence that the connecting peptide of proinsulin is in contact with the solvent-exposed portion of the insulin molecule, figure 3.

Conversely, the decreased immunologic activity of guinea pig insulin in the RIA as compared with the IHIA further strengthens the argument that antibodies operative in the RIA combine with determinants distant from the solvent-exposed portions of the A-chain. Guinea pig insulin has a greater number of substituted residues on the aggregation surfaces and in the A-8-9-10 region and fewer substituted residues in the A-chain solvent-exposed portions of the insulin molecule.

The greater immunologic activity we observed when desoctapeptide-insulin and des-Ala-des-Asp-insulin were used as inhibitors in the RIA as against the IHIA suggests that the perturbations of immunologic determinants on the solvent surfaces of desoctapeptide-insulin and des-Ala-des-Asp-insulin are greater than on the aggregation surfaces and in the dimer sites.

Radioimmune Assay Using Des-Ala-Des-Asp-Insulin Antiserum

Des-Ala-des-Asp-insulin exhibits poor immunologic activity with insulin antiserum, table 2. However, insulin reacts quite well with des-Ala-des-Asp-insulin antiserum. this one-way cross-reactivity was previously observed in the immune hemolysis system.[8] It was proposed that this one-way cross-reaction was due to immunologic determinants common to both insulin and des-Ala-des-Asp-insulin that lie in or near the dimer site and induce antibodies when des-Ala-des-Asp-insulin is the antigen. When insulin is the antigen this determinant is not available to immunocompetent cells because it is buried in the insulin-aggregation site. In like manner, the unexpected cross-reaction of desoctapeptide insulin with des-Ala-des-Asp-insulin antisera indicates homologous regions common to both of these derivatives.

Antibodies directed toward determinants in the dimer site are useful for RIA since they interact at sites distant from the solvent surface and the A-14 and A-19 tyrosyl residues that are iodinated in the ^{125}I-iodoinsulin indicator. It was therefore not surprising to note the high immunologic activity of insulin, ^{127}I-monoiodotyrosyl-insulin, and 3-mononitrotyrosyl-insulin in the RIA in which des-Ala-des-Asp-insulin antiserum was used.

In summary, evidence has been presented that highlights the effect of selection of antibody populations from within a given pool of antiserum by various immunologic indicators in immunoassay systems. In addition, the results obtained with des-Ala-des-Asp-insulin antisera underscore the importance of the variety of determinants to which each of the antibody populations in an antiserum is directed. More provocative is the possibility that the specificities of antibody populations in a given antiserum can be identified and the levels of such antibodies quantitated by

employing a variety of indicators in multiple-assay systems to characterize any given antiserum.

ACKNOWLEDGMENTS

This work was supported by grants PHS-GM02049-04 and PH3-AM13441-05, Kroc Foundation, Santa Ynez, California, and the Diabetes Association of Southern California.

REFERENCES

[1]Arquilla, E. R., Miles, P. V., and Morris, J. W. S.: Immunochemistry of insulin. *In* Handbook of Physiology, Section 7. Steiner, D. V., and Freinkel, N., Eds. Endocrinology. Vol. 1. Endocrine Pancreas. Washington, D. C., American Physiological Society, 1972, pp. 159-73.

[2]Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D.: Insulin: The structure in the crystal and its reflection in chemistry and biology. *In* Advances in Protein Chemistry, Vol. 26. Anfinsen, C. B., Jr., Edsall, J. T., and Richards, F. M., Eds. New York, Academic Press, 1972, pp. 279-402.

[3]Morris, J. W. S., Mercola, D. A., and Arquilla, E. R.: Preparation and properties of 3-nitrotyrosine insulins. Biochemistry *9*:3930-37, 1970.

[4]Hamlin, J. L., and Arquilla, E R.: Monoiodoinsulin: Preparation, purification, and characterization of a biologically active derivative substituted predominantly on tyrosine A-14. J. Biol. Chem. *249*:21-32, 1974.

[5]Morrison, M., and Bayse, G. S.: Catalysis of iodination by lactoperoxidase. Biochemistry *9*:2995-3000, 1970.

[6]Bromer, W. W., and Chance. R. E.: Preparation and characterization of desoctapeptide-insulin. Biochim. Biophys. Acta *133*:219-23, 1967.

[7]Slobin, L., and Carpenter, F.: Action of carboxy-peptidase-A on bovine insulin: Preparation of desalanine-desasparagine-insulin. Biochemistry *2*:16-22, 1963.

[8]Brugman, T. M., and Arquilla, E. R.: Circular dichroic and immunologic studies of structure relationships of insulin and derivatives. Biochemistry *12*:727-32, 1973.

[9]Sodoyez, J. C., Sodoyez-Goffaux, F., Goff, M. M., Zimmerman, A. E., and Arquilla, E. R.: (^{127}I) or carrier free (^{125}I) monoiodoinsulin. Preparation, physical, immunological and biological properties and susceptibility to "insulinase" degradation. J. Biol. Chem. *250*:4268-77, 1975.

[10]Arquilla, Edward R., and Finn, Jack: Insulin antibody variations in rabbits and guinea pigs and multiple antigenic determinants on insulin. J. Exp. Med. *118*:55-71, 1963.

[11]Arquilla, E. R., and Finn, J.: Genetic control of combining sites of insulin antibodies produced by guinea pigs. J. Exp. Med. *122*:771-84, 1965.

[12]Arquilla, E. R., Ooms, H., and Finn, J.: Genetic differences of combining sites of insulin antibodies and importance of C-terminal portion of the A chain to biological and immunological activity of insulin. Diabetologia *2*:1-13, 1966.

[13]Herbert, V., Kam-Seng, L., Gottlieb, C. W., and Bleivher, S. J.: Charcoal immunoassay of insulin. J. Clin. Endocrinol. Metab. *25*:1375-84, 1965.

[14]Hodgkin, D. C.: The structure of insulin, Diabetes *21*:1131-50, 1972.

[15]Gelewitz, E. W., Riedeman, W. L., and Klotz, I. N.: Some quantitative aspects of the reaction of diazonium compounds with serum albumin. Arch. Biochem. Biophys. *53*:411-24, 1954.

[16]Tabachnick, M., and Sobotka, H.: Azoproteins: II. A spectrophotometric study of the coupling of diazotized arsanilic acid with proteins. J. Biol. Chem. *235*:1051-54, 1960.

[17]Rosa, U., Massaglia, A., Pennisi, F., Cozzani, I., and Rossi, C. A.: Effect of the insulin iodination on the reactivity of the inter-chain disulphide bonds towards sodium sulphite. Biochem. J. *103*:407-12, 1967.