Insulin and Anti-Insulin Antibody Interaction

Evidence for the Formation of 7 S and 10 S Structures

KARL-GEORG PETERSEN, MAX-JÜRGEN STORCH, KRISTINA ROTHER, THOMAS LICHT, AND LOTHAR KERP

SUMMARY

The clearing of monoclonal and polyclonal anti-insulin antibodies from homogenous solutions at 100,000 × *g* was used to estimate the size of soluble insulin-antibody complexes at physiologic concentrations. Monoclonal antibodies cleared as a uniform population of 6.6 S independent of the insulin concentration. Polyclonal antibodies cleared as 6.6 S monomers at saturation and as 10 S particles when the amount of insulin bound decreased, suggesting that a soluble complex with two antibodies was formed. An increase of the affinity and a decrease of antibody valency can be related to the complex formation. The binding affinity of polyclonal sera depends on the composition of the affinities of the IgG monomers and on their ability to form 10 S complexes. The formation of insulin-antibody dimers precludes cross-linking and precipitation. Both types of insulin-antibody complexes have been found in the sera from patients treated with bovine insulin. DIABETES 1985; 34:799–802.

Nonprecipitating anti-insulin antibodies are observed during the treatment of patients and after the immunization of animals with insulin. Many other antigens are precipitated because the bivalent antibodies cross-link the binding sites on separate molecules (lattice theory). It is not known why some larger antigens do not precipitate.[1] In the case of insulin, several epitopes have been found.[2] Since one binding site comprises only 5–6 amino acids,[3] insulin may bind to more than one IgG molecule. An interpretation of the nonprecipitating systems[1] assumes that the antibodies bind preferentially with both of their combining sites to determinants on the same antigenic molecule precluding lattice formation (monogamous binding). We have investigated the valency of insulin and its antibodies and the size of the complexes formed in solution.

From the Department of Medicine, Albert-Ludwigs-Universität, Freiburg, FRG. Address reprint requests to Karl-Georg Petersen, Hugstetter Strasse 55, D-7800 Freiburg, FRG.

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MATERIALS AND METHODS

Mono-^{125}I-A14-Tyr biosynthetic human insulin and unlabeled human insulin were obtained from Eli Lilly and Company, Indianapolis, Indiana. B_1-suberoyl-B_1 bovine insulin dimer was kindly supplied by Dr. Schüttler, Deutsches Wollforschungsinstitut, Aachen, FRG.

Guinea pigs were immunized four times by subcutaneous (s.c.) injections into the neck with 1 U/kg body wt human insulin in complete (1:1) and incomplete (2:1) Freund's adjuvant at weekly intervals. Monoclonal anti-insulin antibodies were produced using previously published methods.[2] Fusions from mouse lymph node cells were used.

Polyclonal antibodies from guinea pigs were purified by affinity chromatography on B_1-suberoyl-B_1 insulin dimer sepharose[4] and on protein-A sepharose.[5] The purity of the preparations was checked with columns of insulin sepharose (1 ml). The insulin binding capacity of the void volume was measured by cellulose adsorption. Insulin antibodies were completely retained on the columns. The IgG content of the void volume was measured by Elisa. Non-anti-insulin IgG contaminations were always <5%. The material was uniform on SDS-Page gel electrophoresis.[6] Immunoelectrophoresis showed that the antibodies were of the y-l-type. Guinea pig and mouse IgG were determined by a double-antibody sandwich Elisa[7] and by reading the OD at 278 nm.

To study insulin binding, labeled human insulin (1, 4, 6, and 10 μU/ml) and 10 μU of labeled insulin with unlabeled insulin (10, 20, 39, 78, 156, 313, 625, and 1250 μU/ml) were incubated in 2-ml samples (0.04 M phosphate buffer, pH 8.0, 0.25% albumin) with monoclonal (150 ng/ml) or polyclonal (125 ng/ml) antibodies or without antibody for 48 h at 4°C. Additional samples with 2500, 5000, and 10,000 μU/ml were used for the cellulose adsorption and polyethylene glycol procedures to measure nonspecific binding.

Cellulose adsorption[8] and polyethylene glycol precipitation[9] were used to measure insulin binding. Insulin binding

TABLE 1
Insulin binding by 100 ng of purified anti-insulin IgG from six individual guinea pig sera*

Method	N	K_1 (L/mol)	K_2 (L/mol)	B_1 (μU)	B_2 (μU)	B total (μU)	Insulin/IgG (mol/mol)
Cellulose	6	1.2×10^{11}	2.8×10^{9}	45	135	180	1.7 ± 0.2
PEG	6	6.0×10^{9}	6.1×10^{7}	110	700	810	8.0 ± 1.1
Anti-IgG	6	0.8×10^{9}	0.7×10^{8}	82	104	186	1.9 ± 0.2
Protein-A	6	9.1×10^{9}	—	205	—	205	2.1 ± 0.1

*Molar ratio at saturation of IgG.
The affinity constants and the concentrations of the binding sites were obtained from binding curves of the mean values by graphic analysis.[11] The molar ratio of bound insulin per IgG was calculated from measurements with insulin excess. 1 ng of IgG (M = 156,000) is equimolar to 1 μU of insulin (M = 5800, 1 mg = 27 U).

curves were also obtained by affinity chromatography of IgG on microcolumns (100 μl) of protein-A sepharose CL 4B and of rabbit anti-guinea pig IgG sepharose 6B. The samples were filtered on individual columns to retain IgG (10 min) and washed with 1 ml buffer. Blanks for nonspecific binding obtained without antibody and with excess of cold insulin (10,000 μU/ml) were not different. Bound and free insulin were separated by ultracentrifugation (100,000 × *g*) in a Beckman Ti 50/2 fixed-angle rotor. Plastic tubes of 40 × 3 mm were used. The small diameter was chosen to prevent convection and stirback. After centrifugation the tubes were cut in the middle and counted. The shift of IgG from the upper to the lower part of the tube was calculated. This clearing of IgG refers to centimeters 5.3–6.7 of the rotor radius.

Samples with IgG standards were run and measured by Elisa to determine the time needed for the clearing of IgG. For calculations, an IgG S value of 6.6 was used to compare the data to the literature.[10]

The clearing time of a solution (t) is related to the K factor of the rotor and to the sedimentation constant of the particles (S): $t = K(1/S)$, where K is constant. The amount of particles cleared from the solution (%B) is proportional to the sedimentation constant (S). The size of the particles can be compared according to:

$$t_1/t_2 = S_2/S_1 = \%B_2/\%B_1,\ S_2 = (\%B_2/\%B_1)\ t_1.$$

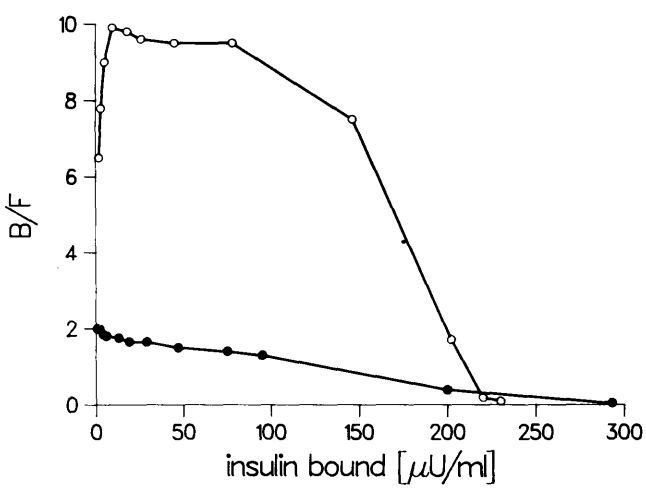

FIGURE 1. Scatchard plots of insulin binding curves obtained by ultracentrifugation (8 h, 100,000 × *g*). Monoclonal antibody (150 ng/ml, affinity 1.1 × 10^9 L/mol, ●——●) and polyclonal antibodies (125 ng/ml, affinities between 0.8 and 11.0 × 10^9 L/mol, ○——○). Affinities were calculated according to $K_1 = B_1/F_1 \times$ (total B − B_1). Insulin concentrations: 1, 4, 6, 10, 20, 30, 49, 88, 166, 323, 635, and 1250 μU/ml.

The number of insulin binding sites per IgG (Table 1) in the filled state was calculated: nonspecific binding is proportional to the insulin concentration if cellulose adsorption, ultracentrifugation, or affinity chromatography are used. The amount of specifically bound insulin is constant at insulin excess with a given antibody concentration. B was calculated by comparing measurements with varying insulin excess.

Student's *t*-test for paired samples was used. Results are expressed as means ± SEM.

RESULTS

The insulin binding of purified anti-insulin IgG from six guinea pig sera was measured with different methods as shown in Table 1. Curvilinear Scatchard plots with two classes of binding sites were obtained when bound and free insulin were separated by cellulose adsorption, by polyethylene glycol (PEG) precipitation, and by affinity chromatography on microcolumns with anti-guinea pig IgG sepharose. An almost linear curve with a uniform type of binding sites was obtained with microcolumns of protein-A sepharose.

One IgG molecule bound two insulins at saturation. The number of binding sites appeared to be overestimated by the PEG method, possibly because the nonspecific binding was slightly nonlinear.

Insulin binding of native insulin, omitting the tracer, was measured after the filtration of samples on protein-A sepharose. Free insulin was determined by radioimmunoassay. Bound insulin was calculated (total − free insulin). No differences were observed in comparison with the tracer technique, excluding artifacts due to the use of labeled insulin.

Insulin binding curves were also obtained after incubation (48 h) at 4°C by ultracentrifugation in a fixed-angle rotor (8 h, 100,000 × *g*). Scatchard plots of experiments with polyclonal anti-human insulin antibodies from guinea pig (125 ng/ml) purified by affinity chromatography and with monoclonal anti-human insulin antibodies from mouse (150 ng/ml), purified on protein-A sepharose, are shown in Figure 1. The concentrations were chosen to include the "paradoxical increase" of the binding curve that is observed at low insulin and high antibody concentrations.

The monoclonal antibody produces a linear plot with an affinity of 1.1 × 10^9 L/mol. A horizontal beginning of the curve at low insulin concentrations (1–5 μU/ml) was observed after 10 h of incubation (not shown). Linearity was always obtained when binding equilibrium was reached by prolonged incubation.

The polyclonal antibodies produce a paradoxical increase at low insulin concentrations that cannot be altered by in-

creasing the incubation time, indicating that a stable equilibrium exists. The decreasing part of the binding curve is commonly used for the determination of binding constants. The purified polyclonal antibodies used in the experiments have only a small amount of low-affinity binding sites. Bound insulin (B) values obtained after quantitative sedimentation of IgG (8 h, 100,000 × *g*) as shown in Figure 1 are calculated as 100% binding at the individual insulin concentrations. The clearing rates (%B) of the samples were measured after centrifugation for shorter intervals of time. A typical experiment is shown in Figure 2. Polyclonal and monoclonal antibodies were run for 60 min in the same rotor. Monoclonal antibodies sediment at a uniform rate that is independent of the insulin concentration. Polyclonal antibodies show a faster sedimentation at low amounts of bound insulin. At saturation the clearing rate is identical to that of monoclonal antibodies and of IgG standards. At a 50-fold excess of antibody (corresponding to the paradoxical increase of the binding curve in Figure 1), there is a slight decrease of the sedimentation velocity indicating dissociation of the larger particles. The sedimentation constant of IgG is 6.6.[10] The calculated constant of the faster particles S_2 is 10.3. A sedimentation constant of 9.9 has been measured for complexes of two IgG molecules.[10]

The clearing rates were measured at 30, 60, and 120 min (Figure 3). The sedimentation constants of the heavy particles calculated from these experiments are 9.8, 10.3, and 9.5, respectively, indicating that the population of the fast particles is homogenous in size.

Purified insulin antibodies (100 ng/ml) from six individual guinea pig sera were incubated with low (20 μU/ml) and high (1260 μU/ml) insulin concentrations. Mean values from six subsequent runs at 100,000 × *g* for 2 and 8 h, respectively, are shown in Table 2. All sera produced 10 S particles at a fivefold antibody excess (mol/mol), which broke down at insulin excess (12.6-fold) to form 7 S particles. The clearing rates were significantly different on the level of individual sera ($P < 0.001$) and for all six sera (73.5 ± 1.0% versus 50.5 ± 0.8% of particles, $P < 0.001$). Sera that did not produce 10 S particles have not been observed.

Insulin antibodies from sera of two patients treated with bovine insulin were examined for the size of complexes formed at insulin and at antibody excess. Dimer and monomer IgG-bovine insulin complexes were observed in both cases (percent of particles cleared: 68 ± 3 versus 52 ± 4 and 67 ± 4 versus 53 ± 3, 10-fold determinations, $2P < 0.05$). The binding of human insulin was too low for calculations.

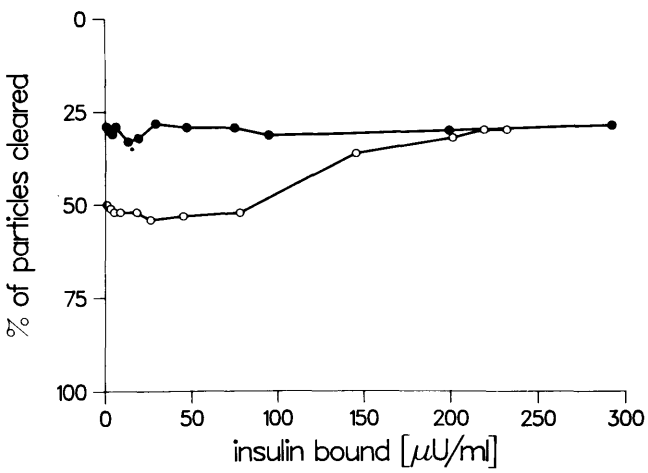

FIGURE 2. Clearing of insulin-antibody complexes by ultracentrifugation (60 min, 100,000 × *g*) at different amounts of bound insulin. 150 ng of monoclonal antibody, ●——●; and 125 ng of polyclonal antibodies, ○——○. Insulin concentrations: see legend to Figure 1.

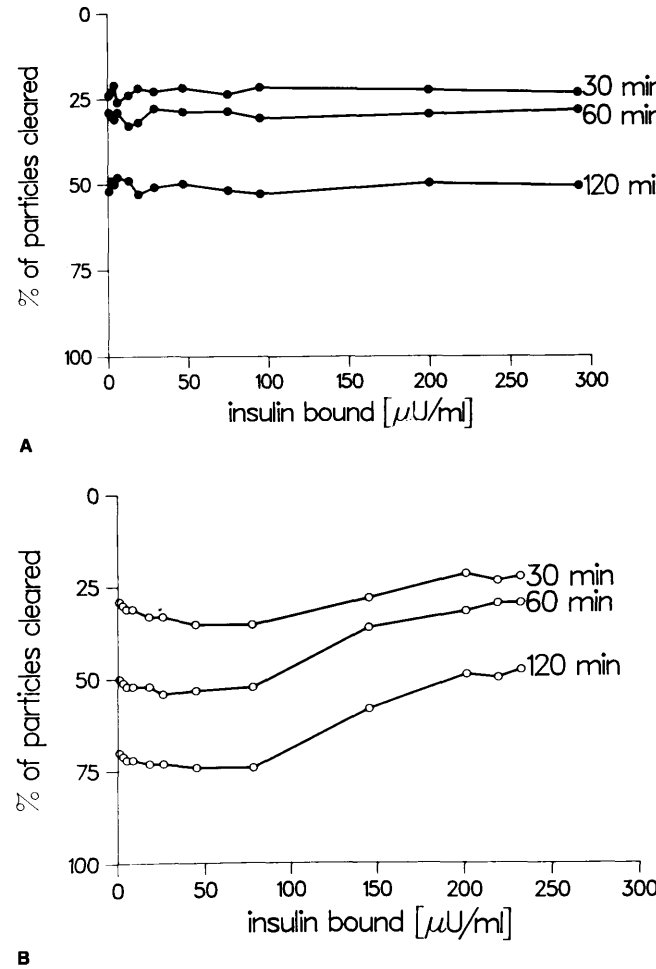

FIGURE 3. Clearing of insulin-antibody complexes by ultracentrifugation (100,000 × *g*) at different amounts of bound insulin after 30, 60, and 120 min. (A) 150 ng of monoclonal antibody, and (B) 125 ng of polyclonal antibodies. Insulin concentrations: see legend to Figure 1.

TABLE 2
Clearance of insulin-antibody complexes from guinea pig after 2 h at 100,000 × g

Serum	N	% of particles cleared	
		20 μU/ml	1260 μU/ml
1	6	73 ± 4	49 ± 3*
2	6	76 ± 3	48 ± 2*
3	6	74 ± 5	53 ± 3*
4	6	70 ± 6	52 ± 3*
5	6	72 ± 5	51 ± 4*
6	6	76 ± 4	50 ± 4*

100 ng/ml of IgG were used. Values are expressed as means ± SEM.
*Significantly different ($P < 0.001$) by Student's *t*-test for paired samples.

DISCUSSION

Since the original observation of nonprecipitating anti-insulin antibodies, a model of a univalent insulin reacting with at least two classes of insulin binding sites with different fixed affinities was used to interpret the binding data.[12] The insulin antibodies were assumed to be bivalent.[8,12] This interpretation of the insulin-antibody interaction was unvalidated because the actual stoichiometry of the binding curves was not known.

The isolation and the quantitation of anti-insulin IgG shows that the antibodies have a binding capacity of two insulins when they are saturated by insulin excess. The presence of several epitopes on the insulin molecule[2] and the size of such binding sites[3] led to the question whether insulin remains univalent if antibodies with different specificity compete for a smaller amount of insulin.

We have thus studied the sedimentation of isolated polyclonal and monoclonal antibodies in relation to the amount of insulin bound. The decision to study the clearing of homogenous solutions at physiologic concentrations in the fixed-angle rotor was made because the equilibrium between insulin and anti-insulin antibodies is not disturbed. IgG, and to a lesser extent insulin, concentrate on the bottom of the tubes at 100,000 × *g*. Using short centrifugation times there are only minor changes of the relative concentrations in the upper parts of the tubes.

The data suggest that monoclonal antibodies occur as a uniform population of monomeric particles independent of the insulin concentration. Polyclonal antibodies sediment as monomers when they are saturated, i.e., two insulins are bound to each bivalent IgG. With lower amounts of insulin bound, increasing amounts of complexes with two IgG molecules are formed until insulin is completely bound to the 10 S IgG complexes. Larger complexes are not observed. The dimeric complexes become unstable at extremely low ratios of insulin to antibody. The latter observation can explain the "paradoxical increase" of insulin binding curves.

Soluble IgG dimers have been observed when the interaction of bivalent symmetrical 2,4-dinitrophenyl (DNP) haptens with rabbit anti-DNP antibodies was studied in the analytic ultracentrifuge.[10]

A ring-shaped structure consisting of two bivalent haptens and two bivalent IgG molecules was formed.[10] In the case of insulin, a variety of epitopes is exposed to the antibodies. Monoclonal antibodies recognize only one specific epitope on each insulin molecule. They are thus unable to form complexes.

Polyclonal antibodies recognize epitopes on insulins bound to antibodies with differing specificity. Insulin plays the role of a bivalent hapten to form a stable structure of two insulins with two different bivalent anti-insulin antibodies. Since the formation of the 10 S particles leads to an increase of affinity, a ring structure must be assumed on account of thermodynamic calculations.[10] Large and small increments of affinity may result, depending on the strain induced by the relative positions of the epitopes on the insulin molecules.

Our data show that the 10 S complexes of insulin and antibodies break down with increasing insulin concentrations. The concomitant loss of binding affinity can be compared to the negative cooperativity observed in insulin receptor systems. Anti-insulin antibodies do not produce site-site interactions on the level of the two binding arms of IgG as results from the linear binding curves of monoclonal antibodies. There is, however, variation in the affinity of these clones.[2] Cooperation is introduced by the formation of circular complexes. An increase of affinity occurs when the molar ratio of bound insulin per IgG shifts from two (saturated IgG monomer) to one (10 S complex). The biologic role of the 7 S and 10 S complexes in human sera has to be evaluated.

The formation of circular complexes between a small antigenic molecule with several different epitopes (insulin) and polyclonal antibodies with differing specificities prevents cross-linking and precipitation of this system in spite of the bivalency of antigen and antibody.

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