Maturation of Immune Response to Insulin

Recruitment of New Epitopes by Antibodies

K.-G. PETERSEN, G. SCHULER, W. HÖSL, M.-J. STORCH, AND L. KERP

SUMMARY

Guinea pigs were immunized with regular insulin in Freund's adjuvant weekly ($N = 6$) or with NPH insulin daily ($N = 10$). The concentrations, the affinities, and the sizes of the insulin–immunoglobin G (IgG) antibody complexes were determined by ultracentrifugation. During the immune response to insulin, 7-S complexes were observed after 3 wk, and 10-S complexes were observed after 4 wk ($2P < .05$ in each group).

"7-S" antibodies were transferred into immunized guinea pigs before the formation of measurable antibody concentrations. The development of 10-S structures was enhanced in outbred Pirbright white guinea pigs ($2P < .05$, $N = 6$) and in inbred strain II I.b.m. guinea pigs ($2P < .05$, $N = 7$). Insulin may act as a monovalent antigen (7 S) after 3 wk and become bivalent after 4 wk (10 S). The change in valency is enhanced by antibody transfer. It reflects the formation of antibodies recognizing new epitopes on insulin.
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Much of the data presented over the years about insulin antibodies has been based on the assumption of a monovalent insulin[1] forming antibody complexes with one immunoglobin G (IgG) molecule (7 S). Ultracentrifugal studies,[2,3] however, have shown that insulin is bivalent. The respective antibodies must occur as suitable pairs to fit to two different and sufficiently distant epitopes on the small insulin molecule. The presence of insulin-antibody complexes with two IgG molecules (10 S) thus reflects antibody diversity.

In our study, a follow-up of the immune response to insulin shows a progression from 7-S to 10-S complexes during the antibody maturation process. Different activation times of the respective B-lymphocyte clones could initially result in an antibody population with uniform specificity. 7-S complexes would be followed by 10-S complexes, with increasing numbers of different antibody-producing cells. Alternatively, antibody diversity could result from the interaction of insulin with early antibodies that obscure primary and expose secondary epitopes. The latter hypothesis is tested by the transfer of early antibodies during the immunization process.

From the Department of Endocrinology, Albert-Ludwigs-Universität, Freiburg, Federal Republic of Germany.
Address reprint requests to Karl-Georg Petersen, Hugstetter Strasse 55, D-7800 Freiburg, FRG.
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MATERIALS AND METHODS

Unlabeled and mono-^{125}I-A 14-Tyr semisynthetic human insulin were kindly supplied by Hoechst AG (Frankfurt, FRG). Outbred male Pirbright white guinea pigs were purchased from Dr. Ivanovas (Kiessleg, FRG). Inbred female specific-pathogen–free (SPF) strain II I.b.m. guinea pigs were a generous gift of the Biological and Medical Research Institute (Füllinsdorf, Switzerland).

To follow the immune response, six Pirbright white guinea pigs (body wt, 344 ± 33 g) were immunized by subcutaneous injections into the neck with 1 U/kg body wt human regular insulin in complete (1 ×) and incomplete Freund's adjuvant at weekly intervals. Aliquots of insulin with Freund's adjuvant were prepared and kept at −20°C. Ten Pirbright white guinea pigs (body wt, 354 ± 23 g) received daily subcutaneous injections of an equal dose of human NPH insulin. Blood was drawn weekly by cardiac puncture (24-gauge needle).

In the antibody-transfer experiments (see Fig. 2) immunization with regular insulin in Freund's adjuvant weekly was started with the donor animals. The recipients followed with a delay of 8 days (1 wk plus 1 day for preparation of IgG). After 3 wk, blood was drawn from the donor animals in the morning. Individual IgG was isolated on protein A sepharose columns.[4] The eluates (0.1 M citric acid adjusted to pH 3.5 with 0.2 M Na_2HPO_4) were brought to pH 7.4 with saturated Tris. Three-milliliter aliquots were diluted with 3 ml of saline and injected intraperitoneally into the recipients in the evening. The donors were reimmunized 2 h after bleeding, and the recipients were reimmunized the next day. Equal amounts of blood were drawn from donor and recipient animals with

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TABLE 1
Concentrations, high-affinity constants (calculated[1] and determined[2] by Scatchard plot), and clearing rates

Anti-insulin antibodies	Human regular insulin with adjuvant weekly (N = 6) 3 wk		Human regular insulin with adjuvant weekly (N = 6) 4 wk	Human NPH insulin daily (N = 10) 3 wk		Human NPH insulin daily (N = 10) 4 wk
Concentration (μg/ml)	24.2 ± 6.1	$2P < .05$	39.1 ± 9.8	150.4 ± 7.3	$2P < .02$	178.2 ± 9.1
High-affinity 1 (1/mol · 10^9)	0.6 ± 0.1	$2P < .05$	2.8 ± 0.5	0.8 ± 0.1	$2P < .05$	2.2 ± 0.4
High-affinity 2 (1/mol · 10^9)	0.9 ± 0.3	$2P < .05$	3.4 ± 0.7	1.1 ± 0.2	$2P < .05$	3.6 ± 0.5
Clearing rate [2/8 h (%)]	51.2 ± 5.9	$2P < .05$	67.2 ± 2.1	58.9 ± 1.0	$2P < .05$	67.1 ± 1.1

Values are for antibodies during immunization of outbred male Pirbright white guinea pigs with insulin (see legend of Fig. 1). Student's t test for paired samples was used. Values are means ± SEM.

equivalent timing. Four groups of six outbred male Pirbright white guinea pigs were used for the first antibody-transfer experiment: untreated control donors (body wt, 267 ± 23 g), immunized recipients for control IgG (body wt, 241 ± 32 g), immunized donors (body wt, 243 ± 15 g), and immunized recipients for insulin antibodies (body wt, 255 ± 41 g). The reproduction of the experiment in inbred female SPF strain II I.b.m. guinea pigs was performed with seven immunized donors (body wt, 363 ± 34 g) and seven immunized recipients (body wt, 347 ± 27 g).

For insulin-binding studies, individual IgG eluates from protein A were dialyzed (0.04 M phosphate buffer, pH 8.0). These antibodies were not further purified by affinity chromatography. An insulin matrix might interfere with the diversity of the sera because antibodies to some epitopes could preferentially resist elution from the column.

The methods used in binding studies with highly purified antibodies were applicable.[3] Anti-insulin antibodies purified previously by affinity chromatography on B_1-suberoyl-B_1 bovine insulin-dimer sepharose[5] and on protein A sepharose[4] were reconstituted with IgG from controls to the amount removed by purification with B_1-B_1 insulin. These small amounts of IgG did not interfere with the binding measurements in the ultracentrifuge.

The binding studies were uniformly done with diluted antibodies at a final insulin-binding capacity of 200 ± 40 μU/ml

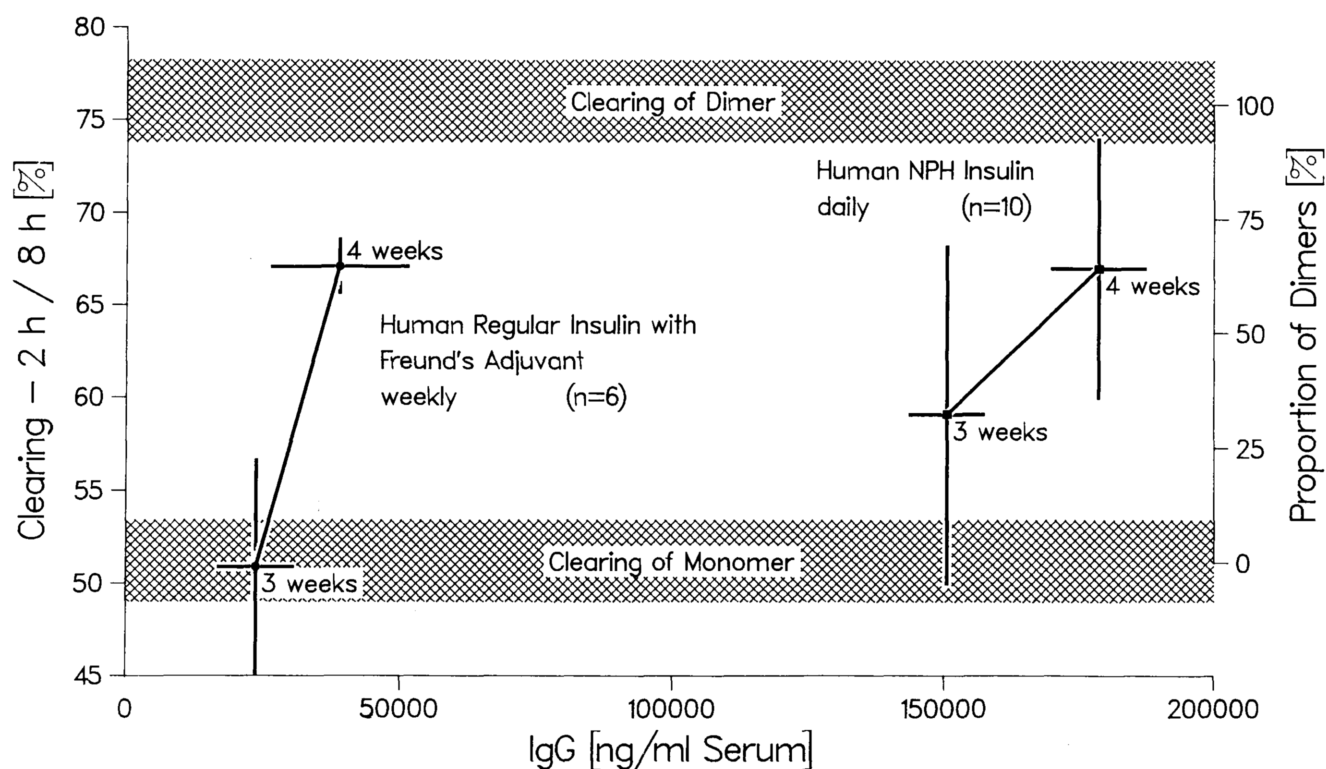

FIG. 1. Clearing rates of insulin-antibody complexes at antibody excess 3 and 4 wk after immunization of Pirbright white guinea pigs with human regular insulin (0.1 U/kg body wt) and Freund's adjuvant weekly or with an equal dose of human NPH insulin daily. Incomplete clearing (100,000 × *g*, 4°C) after 2 h is compared with complete clearing after 8 h. Proportion of 10-S particles is calculated from clearing rates. *Abscissa* gives amount of insulin antibodies calculated at insulin excess (means ± SEM). Male guinea pigs were used [body wt, 344 ± 33 g (*N* = 6) and 354 ± 23 g (*N* = 10)].

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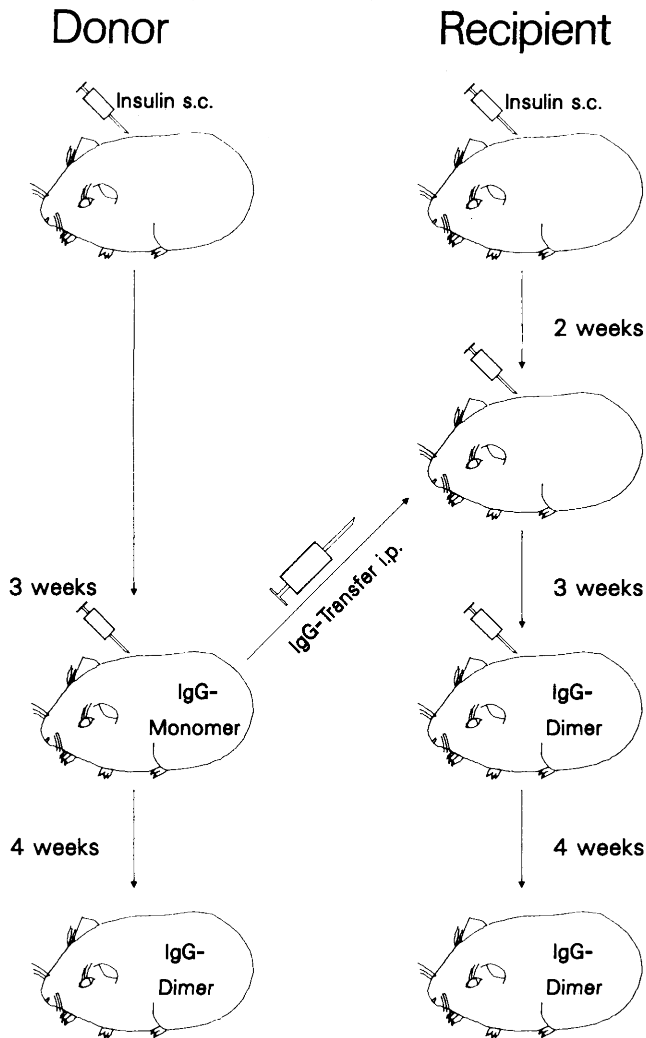

FIG. 2. Antibody transfer during immunization with insulin in Freund's adjuvant (0.1 U/kg body wt) weekly. Data from this experimental setup are given in Table 2.

when the sera of different groups of animals were compared. Ten microunits of labeled insulin and unlabeled insulin (0, 10, 20, 39, 78, 156, 313, 625, and 1250 μU) were added and incubated in 1-ml samples (0.04 M phosphate buffer, pH 8.0, 0.25% human serum albumin) for 12 h at 4°C. Blanks without antibody and the samples with 10 and 1260 μU/ml were run in triplicate.

Ultracentrifugation of the clear solution (100,000 × *g*, 4°C) in a Beckman Ti 50/2 fixed-angle rotor (Beckman, Fullerton, CA) was used to separate bound and free insulin.[3] After centrifugation, the plastic tubes (40 × 3 mm) were cut in the middle and counted. The shift of the insulin-antibody complexes from the upper to the lower part of the tube was measured. After complete clearing of IgG (8 h), the antibody concentrations were calculated from the binding capacity at insulin excess as described previously.[3] The affinities were measured by graphic analysis of the binding curves[6] and calculated from the concentrations of insulin, antibody, and insulin-antibody complexes

$$k = \frac{B'}{F'(B - B')} (1.63 \times 10^{11})$$

where B′ is insulin bound at 10 μU/ml; F′ is free insulin at 10 μU/ml; B is insulin bound at 1260 μU/ml. The clearing rate was calculated comparing incomplete clearing after 2 h with complete clearing

$$a_2 = \frac{l_2}{u_2} \qquad a_8 = \frac{l_8}{u_8}$$

$$B_8 = \frac{L_8 - U_8 a_8}{A} = B_2 \qquad \text{bound insulin}$$

$$F_8 = \frac{L_8 + U_8}{A} - B_8 = F_2 \qquad \text{free insulin}$$

$$F_{L_2} = F_2 \frac{l_2}{l_2 + u_2} = F_2 \frac{a_2 u_2}{a_2 u_2 + u_2} = F_2 \frac{a_2}{a_2 + 1} \qquad \text{free insulin in lower half}$$

$$Sed_2 = \frac{L_2}{A} - F_{L_2} - \frac{B_2}{2} \qquad \text{bound insulin shifted from upper to lower half}$$

where u_2 is count in upper half of tube, insulin only, after 2 h; l_2 is count in lower half of tube, insulin only, after 2 h; u_8 is count in upper half of tube, insulin only, after 8 h; l_8 is count in lower half of tube, insulin only, after 8 h; U_2 is count in upper half of tube, insulin and antibodies, after 2 h; L_2 is count in lower half of tube, insulin and antibodies, after 2 h; U_8 is count in upper half of tube, insulin and antibodies, after 8 h; L_8 is count in lower half of tube, insulin and antibodies, after 8 h; and A is specific activity. The Svedberg constants were calculated by comparison of the clearing rates with control IgG.[3]

Comparison of two different particles

$$t_{c_1} = \frac{t}{C_1} \qquad t_{c_2} = \frac{t}{C_2}$$

$$S_1 = \frac{k}{t_{c_1}} = \frac{k}{t} \times C_1 \qquad S_2 = \frac{k}{t_{c_2}} = \frac{k}{t} \times C_2$$

$$\frac{S_1}{S_2} = \frac{\frac{k}{t} \times C_1}{\frac{k}{t} \times C_2} = \frac{C_1}{C_2}$$

where t is centrifugation time (const, 2 h), k is rotorconstant, Sed is sedimented particles after t, Tot is total amount of particles, C = Sed/Tot, $t_c = k/C$ (t_c is the time for total clearing), S is Svedberg constant for a known particle, $t = k/S_c \leftrightarrow S_c = k/t$. Student's t test for paired samples was used. Results are expressed as means ± SEM.

RESULTS

Two weeks after the initial immunization of 6 Pirbright white guinea pigs, no antibodies (<10 ng/ml) were found after human insulin with Freund's adjuvant weekly, but 4 out of 10 Pirbright white guinea pigs injected with NPH insulin daily had just-detectable antibody levels. During the 3rd wk, measurable anti-insulin antibody concentrations (>10 ng/ml) developed in all guinea pigs (Table 1 and Fig. 1). Daily injec-

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TABLE 2
Concentrations, high-affinity constants (calculated[1] and determined[2] by Scatchard plot), and clearing rates

Anti-insulin antibodies	Pirbright (N = 6)			II l.b.m. (N = 7)	
	IgG recipients	Antibody donors	Antibody recipients	Antibody donors	Antibody recipients
Concentration (μg/ml)	16.4 ± 4.3	17.2 ± 5.0	27.9 ± 4.1	35.1 ± 2.6	4.6 ± 0.4
	└── NS ──┘	└── $2P < .02$ ──┘		└── $2P < .001$ ──┘	
High affinity 1 (1/mol · 10^9)	1.0 ± 0.3	0.6 ± 0.2	2.0 ± 0.3	3.3 ± 0.2	4.9 ± 0.5
	└── NS ──┘	└── $2P < .05$ ──┘		└── $2P < .05$ ──┘	
High affinity 2 (1/mol · 10^9)	1.1 ± 0.4	0.7 ± 0.3	3.1 ± 0.3	3.5 ± 0.3	5.2 ± 0.6
	└── NS ──┘	└── $2P < .05$ ──┘		└── $2P < .05$ ──┘	
Clearing rate [2/8 h (%)]	56.8 ± 1.8	55.1 ± 2.1	65.0 ± 0.9	56.0 ± 0.6	67.4 ± 0.9
	└── NS ──┘	└── $2P < .05$ ──┘		└── $2P < .05$ ──┘	

Insulin-antibody complexes in male outbred Pirbright white guinea pigs (body wt: 241 ± 32 g, IgG recipients; 243 ± 15g, antibody donors; 255 ± 41 g, antibody recipients) and inbred female II l.b.m. guinea pigs (body wt: 363 ± 34 g, antibody donors; 347 ± 27 g, antibody recipients). Experimental setup is described in Fig. 2. Results are expressed as means ± SEM. Values ($2P$) in parentheses denote significant differences ($2P$).

tions of human NPH insulin produced higher titers than the weekly injection of regular human insulin with Freund's adjuvant ($2P < .001$). The high affinities of the antibodies were comparable after 3 wk in both groups. The insulin-antibody complexes sedimented at antibody excess with a velocity comparable to control IgG, i.e., as monomeric (7-S) complexes of insulin and IgG (Fig. 1).

During the 4th wk, the antibody concentrations increased by 38% in the group of guinea pigs injected with insulin and Freund's adjuvant weekly and by 16% in the animals treated with daily injections of NPH insulin. Comparable increases of the affinities were observed. The clearing rate of the insulin-antibody complexes at antibody excess rose to 67% in both groups, indicating the additional formation of larger (10-S) insulin-antibody complexes. The proportion of 10-S particles necessary to produce the respective clearing rates can be seen in Fig. 1. The high antibody affinity and the clearing rate, i.e., the mean size of the complexes, were correlated in each group ($P < .05$).

With continued weekly immunization, a final clearing rate of 76 ± 2% (N = 6) was reached after 7 wk. An increase in the binding capacity of the antibodies (4th wk) from 200 to 5000 μU/ml at an insulin concentration of 1000 μU/ml resulted in a clearing rate of 74 ± 3% (N = 6).

The influence of early antibodies on the elaboration of antibodies with suitable specificity to form dimeric (10-S) insulin-antibody complexes was tested by antibody transfer as shown in Fig. 2. The antibody population formed in the recipients was compared with the donor antibodies (Table 2).

Male outbred Pirbright white guinea pigs received IgG with a total binding capacity of 50 ± 6 mU of insulin. The production of antibodies was unchanged, but there was a significant increase in the clearing rate of the insulin-antibody complexes in the recipients (Table 2). Inbred female strain II l.b.m. guinea pigs received IgG with a total binding capacity of 140 ± 20 mU of insulin. The production of antibodies in these recipients was significantly inhibited (Table 2). The high affinity increased nevertheless ($2P < .05$). The clearing rate rose significantly from 56% in the donors to 67% in the recipients. Injection of control IgG did not alter the amount, the affinity, or the clearing rate of the antibodies formed in Pirbright white guinea pigs.

The recipients had received ~5–10% additional IgG from the donors. The increase of dimer formation might have resulted from the mixture of the sera of donor and recipient. This possibility was tested in vitro by mixing the sera of two donors (9 parts plus 1 part at 15 and 21 combinations, respectively). No increase in the clearing rate was observed [56 ± 3% (N = 36) vs. 55 ± 4% (N = 13)].

DISCUSSION

Two different ultracentrifugation techniques gave evidence that insulin acts as a bivalent antigen in guinea pig and human sera.[2,3] An upper limit of the clearing rate (76%) and the uniform clearing with time[3] suggest that complexes with two IgG molecules (10 S) are formed at antibody excess.

The size of insulin is comparable with that of the binding arm of IgG (35 Å). Steric hindrance limits the simultaneous binding of antibodies to two sites. A diversity of antibodies, which allows the binding to determinants opposite to one another on the insulin molecule,[2] is a prerequisite for the formation of 10-S insulin-antibody complexes.

The first IgG antibodies to insulin that appear during the 3rd wk of immunization can only form 7-S complexes. With continued immunization, an increase of antibody diversity occurs so that 10-S particles are formed. The immune response to insulin proceeds with persisting antigenic pressure until insulin is functionally bivalent. It depends on the completeness of this process and on the concentration of insulin and antibodies in the test system whether 7-S, 10-S, or a mixture of both particles is observed.

A sequential development of 7-S and 10-S insulin-antibody complexes is in contradiction to the rule that the most powerful antibodies are selected during the immune response leading to a decrease of antibody diversity.[7,8] The concentrations of the early antibodies in the sera of the guinea pigs and the half-life of insulin in Freund's adjuvant (5 h) suggested that exogenous insulin would be almost completely bound to antibodies after 3 wk. Whether the early IgG antibodies interfere with the antigenic properties of insulin by antibody transfer was tested. Outbred and inbred guinea pigs produced 10-S instead of 7-S complexes when they had received early (7-S) IgG insulin antibodies before the third immunization. We conclude that the transfer of antibodies has induced the involvement of new epitopes into the immune response to insulin. The appearance of 10-S

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complexes depends on early antibodies but not on the activation times of the different B-lymphocyte clones.

Alternatively, anti-idiotypic antibodies[9] could be responsible for the formation of larger complexes because the antibodies were not purified on an insulin matrix. However, 7 days is too short for the induction of significant amounts of anti-idiotypic antibodies in the sera of guinea pigs. On the other hand, these antibodies bind to the same site that insulin does. The method used here follows the clearing of antibody-bound labeled insulin. After displacement of insulin by anti-idiotypic antibodies, most of these complexes would not be measured.

The idea that antibodies play a regulatory role in the immune response is not new[10–12] but is difficult to prove without a marker of antibody diversity as used here. Antigenic competition[13] may explain how early insulin antibodies involve new epitopes into the immune response: specific epitopes on the antigen are obscured by antibody binding. The antigen exhibits only residual epitopes, which no longer compete for binding to the immune cells with the original determinants. The recognition of new epitopes is thus facilitated. The diversity of antibodies on the cellular level is translated into the diversity of the immune response with the assistance of an antibody-mediated control of recognized epitopes. The inhibition of antibody formation in the strain II I.b.m. guinea pigs after transfer of a high dose of antibodies is also compatible with the concept of antigenic competition.

Steric hindrance favors an almost opposite position of the binding sites on an insulin molecule.[2,3] The first epitope recognized will thus largely determine the second antibody binding site. Because the mixing of early (7-S) antibodies from two donors did not result in the formation of 10-S particles, a low interindividual variation in the pattern of epitopes recognized initially must be assumed. "Leading" epitopes could exclude surface areas of insulin from becoming antibody binding sites. The biologically active site is thus not necessarily involved as an epitope. This could explain why antibody-mediated insulin resistance is rare.

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