Recognition of Human Insulin and Proinsulin by Monoclonal Antibodies

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SUMMARY

High-affinity monoclonal antibodies (MAB) were obtained from lymph node cell fusions. Affinities ranging from 0.8 × 10^9 L/M to 5.2 × 10^9 L/M were calculated from binding studies with monoiodinated human, bovine, and porcine insulins and human proinsulin.

Two monoclonal antibodies were specific for human insulin, recognizing an epitope involving the amino acid B-30 (Thr). Another two monoclonal antibodies were bound to the C-terminal end of the B-chain near B-30.

The B-chain-specific monoclonal antibodies did not bind human proinsulin. One monoclonal antibody recognized the A-chain loop in the positions A-8 to A-10. This antibody bound also to human proinsulin. It was concluded that the A-chain loop is exposed on the surface of proinsulin, while the C-terminal B-chain is not available for binding.

The study shows that monoclonal antibodies can be used to characterize structures of insulin and proinsulin. In contrast to x-ray studies, the molecules can be used at low concentrations in soluble form. It is suggested to use monoclonal antibodies for the screening of atypical insulins in the serum of diabetic patients and for the further refinement of insulin and proinsulin measurements. DIABETES 1985; 34:808–11.

The immune response to insulin is known to be under MHC-linked Ir-gene control.[1] The response to a species-specific insulin is dependent on the presence of a suitable carrier determinant on the molecule.

The specificity of recognition is due to helper T-cells and is not necessarily reflected by the antisera that are the products of a population of B-cells. If an animal responds to an antigen that it can recognize, its immune serum often shows cross-reactions with related antigens. In the case of insulin, most sera show cross-reactions,[1] some species-specific antisera have however been described.[2] The relative "nonspecificity" of most antisera can be circumvented by the use of monoclonal antibodies (MABs).[3]

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MATERIALS AND METHODS

Materials. Mono-^{125}I-A14-Tyr human proinsulin and mono-^{125}I-A14-Tyr human insulin were a gift from Dr. Bruce Frank, Lilly Research Laboratories, Indianapolis, Indiana. Mono-^{125}I-A14-Tyr porcine and mono-^{125}I-A14-Tyr bovine insulin were obtained from Hoechst, Frankfurt, FRG. All tracer insulins were purified by reverse-phase HPLC. Unlabeled bovine and porcine insulins purified by gel chromatography (Hoechst, Frankfurt, FRG) and unlabeled biosynthetic human insulin[4] and human proinsulin[5] (Eli Lilly and Company, Indianapolis, Indiana) were used. Fetal calf serum, Dulbecco's minimal essential medium, and Freund's complete adjuvant were purchased from Gibco, Grand Island, New York.

Animals. Female BALB/c mice from the Max-Planck-Institut für Immunbiologie (Freiburg, FRG) were used for immunization and fusion. Ten mice were immunized with 20 μg biosynthetic human insulin (Lilly) in 50 μl Freund's complete adjuvant in the rear footpads. On day 14 after immunization, the inguinal lymph nodes were prepared and used for fusion. Immunization for spleen cell fusions were done with two intraperitoneal (i.p.) injections of 20 μg antigen in Freund's complete adjuvant at an interval of 4 wk.

Production of MABs. Spleen cells and lymph node cells were fused with the myeloma line X63 Ag 8.6.5.3. as described.[6] Of 144 cultures tested (from a lymph node fusion), 12 produced antibodies binding labeled human insulin. Five of these were cloned by limiting dilution. The cloned hybridoma antibodies were characterized as IgG 1 using a solid-phase assay previously described.[7] Hybridoma culture supernatants were purified by protein-A Sepharose Cl4b (Pharmacia, Uppsala, Sweden). The MABs differed in their binding to protein-A. Differing amounts of the antibodies were lost in the void volumes.

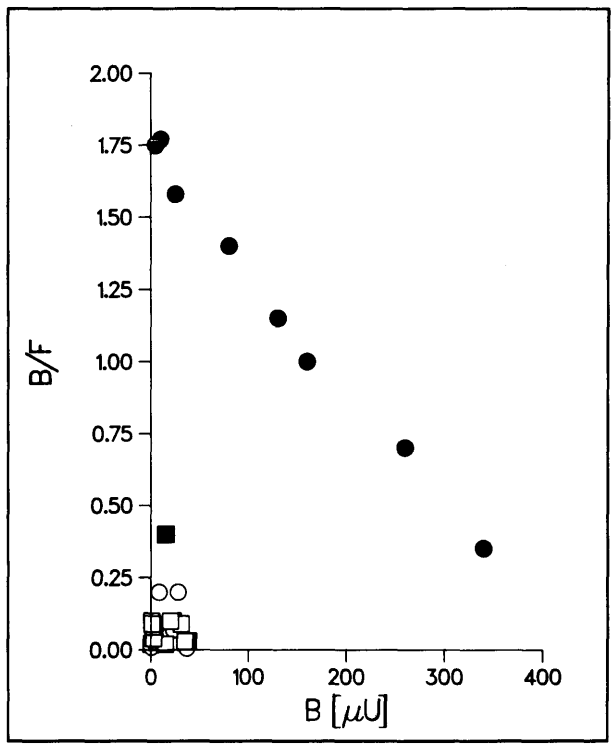

FIGURE 1. Scatchard plots of binding curves from monoclonal antibody 2 with human insulin (●), porcine insulin (○), bovine insulin (■), and human proinsulin (□). Binding of human insulin, porcine insulin, and human proinsulin was seen with comparable affinities; no binding of bovine insulin was noted.

Solid-phase screening assay for anti-insulin antibodies. Polyvinylchloride microtiter plates were coated with 100 μl of a 1:100 dilution of rabbit anti-mouse immunoglobulins (Dako, Kopenhagen, Denmark) in phosphate-buffered saline (PBS) for at least 1 h. Plates were then washed three times with PBS and blocked for 30 min with 200 μl of PBS containing 3% bovine serum albumin (BSA). After three washings, the hybridoma supernatants (100 μl) were added for 1 h. After washing, labeled human insulin was added (about 10×10^3 cpm/well) for 1 h. The plates were washed, cut, and counted.

Solid-phase enzyme immunoassay for mouse immunoglobulins. Flexible PVC microtiter plates were coated with 100 μl of a 1:100 dilution of rabbit anti-mouse immunoglobulins in PBS for 1 h. After washing three times with PBS, the plates were blocked with a 3% BSA solution for 30 min. MAB-containing supernatants were added for 1 h (100 μl). The plates were then washed. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) were added (1:100 dilution) for 1 h. After three washings with PBS, the substrate of peroxidase (*o*-phenylenediamine, 0.55 mg/ml) was added for 15 min and the color reaction stopped with 1 M H_2SO_4 (50 μl). Optical density at 492 nm was read on a Kontron SLT 210 reader.

Binding studies. Cellulose adsorption,[8] polyethylene glycol precipitation,[9] and ultracentrifugation[10] were used to obtain insulin-antibody binding curves. Additionally, bound and free insulin were separated by affinity chromatography of IgG on microcolumns (200 μl) of protein-A Sepharose Cl4 b as previously described.[11]

Labeled (10 μU/ml) and serial dilutions of unlabeled insulin (0, 10, 20, 39, 78, 156, 313, 625, and 1250 μU/ml) were incubated with purified MABs (100 ng) in 1 ml of phosphate buffer (0.04 M, pH 8.0, 2.0% albumin) for 24 h at 4°C. Blanks for nonspecific binding were run without antibody.

RESULTS

Fusions of spleen cells from mice immunized with human insulin resulted in weakly positive insulin binding hybridoma antibodies as judged by screening experiments.

In contrast, strongly positive hybridoma supernatants were obtained from lymph node cell fusions. It has been described that the main immune response to insulin occurs in lymph nodes.[12] These hybridomas were cloned twice and the resulting MABs were characterized by binding studies.

Binding studies were performed by four methods. The cellulose method separates free and bound insulin by adsorption of the free insulin to cellulose; no binding curves were obtained. The MABs rested in the supernatant (as judged by enzyme immunoassay of mouse immunoglobulins), while insulin was completely adsorbed by cellulose. Polyethylene glycol did not precipitate the highly purified MABs sufficiently when human serum was used as carrier protein. The MABs differed in their binding to protein-A. Binding curves obtained by separating antibodies and free insulin on protein-A Sepharose columns could not be compared. Reproducible insulin binding was measured after sedimentation of immunoglobulin by ultracentrifugation (100,000 × *g*, 8 h).

Figure 1 shows the Scatchard plots of insulin binding experiments of MAB 2 with labeled human, porcine, and bovine

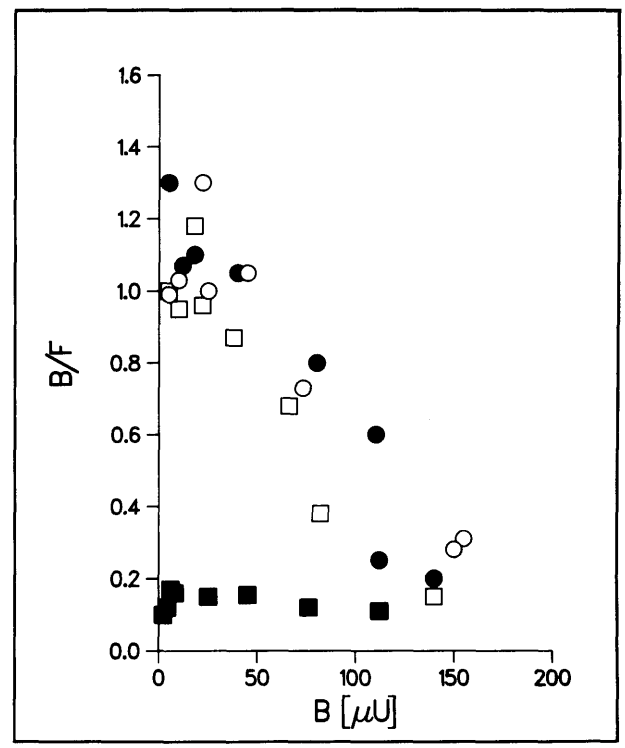

FIGURE 2. Scatchard plot of binding curves from monoclonal antibody 5 with human insulin (●), porcine insulin (○), bovine insulin (■), and human proinsulin (□). This antibody is specific for human insulin.

TABLE 1
Affinity constants of five monoclonal antibodies to different insulins [l/M]

MAB	Human insulin		Porcine insulin		Bovine insulin		Human proinsulin	
1	1.8×10^9	(0.99)*	$<1 \times 10^6$	(—)	$<1 \times 10^6$	(—)	$<1 \times 10^6$	(—)
2	1.3×10^9	(0.96)	1.3×10^9	(0.91)	$<1 \times 10^6$	(—)	0.9×10^9	(0.98)
3	2.5×10^9	(0.91)	0.8×10^9	(0.88)	0.7×10^9	(0.95)	$<1 \times 10^6$	(—)
4	5.2×10^9	(0.97)	3.3×10^9	(0.92)	2.4×10^9	(0.98)	$<1 \times 10^6$	(—)
5	0.8×10^9	(0.94)	$<1 \times 10^6$	(—)	$<1 \times 10^6$	(—)	$<1 \times 10^6$	(—)

The binding curves were obtained by ultracentrifugation.
*Coefficient of correlation of Scatchard plots.

insulins, and human proinsulin. Linear Scatchard plots were obtained over a wide range of insulin concentrations (1–1260 μU/ml). MAB 2 bound human insulin, porcine insulin, and biosynthetic human proinsulin with comparable affinities (see Table 1). No binding was observed with bovine insulin.

Figure 2 shows the Scatchard plots of MAB 5, which recognizes human insulin, but not porcine insulin, bovine insulin, and human proinsulin. The affinity constants of the binding experiments are summarized in Table 1.

The binding properties of MAB 1 were similar to those of MAB 5. The binding specificity for human insulin suggests that the amino acid in position B-30 (Thr) is part of the epitope recognized. An epitope near B-30 must be assumed for MABs 3 and 4, because the affinity constants for porcine and bovine insulins were reduced in comparison to those of human insulin. None of the MABs with specificity for the terminal B-chain bound biosynthetic human proinsulin.

DISCUSSION

Five MABs with high affinity to human insulin have been analyzed with regard to their specificity. Binding data were obtained by ultracentrifugation of samples incubated with human insulin, human proinsulin (C-peptide connecting the amino acids B-30 and A-1), porcine insulin (B-30: alanine instead of threonine), or bovine insulin (B-30: alanine; A-8: alanine instead of threonine; A-10: isoleucine instead of valine).

Four of the MABs exhibited specificity for the C-terminal end of the B-chain. In two of these the threonine in position B-30 was an indispensable part of the epitope. The terminal end of the human insulin B-chain that differs in the position B-30 from mouse insulin I and in the positions B-29 and B-30 from mouse insulin II appears to be an essential antigenic determinant in mice.

One MAB was specific for the A-8 to A-10 region of human insulin, which does not differ from mouse insulin in these positions; it is thus autoreactive.

Others have prepared 15 MABs by immunization with bovine insulin, three with human insulin.[3] None of the three antibodies was specific for human insulin; they showed cross-reaction especially with porcine insulin. The affinities (2×10^7–3×10^8 L/M) were comparable. One MAB recognized the human A-chain loop as did our MAB 2, and two others were specific for the bovine A-chain loop, indicating that this region of the insulin molecule is an essential epitope.

While 4 of our MABs were specific for the C-terminal B-chain, none of the 18 MABs described[3] was directed to this region.

In the proinsulin molecule, the C-peptide chain connects positions A-1 and B-30 of the insulin molecule. X-ray studies show that the three-dimensional structure of the insulin moiety of proinsulin is identical to that of insulin.[13] Proinsulin can be regarded as an A-1, B-30 modification of insulin. Parts of the surface can be covered by the C-peptide.

One interpretation is that the terminal end of the B-chain is covered by C-peptide, because none of the four B-chain-specific MABs bound to human proinsulin. Another major possibility is that the free carboxyl group of the C-terminus is required for binding.

The region A-8–A-10 is exposed on the surface of proinsulin, since the MAB with specificity for the A-chain loop bound to human insulin and proinsulin with the same affinity. The specificity to this epitope results from the fact that this MAB is unable to recognize bovine insulin (with different amino acids in positions A-8 and A-10).

This study shows that MABs can be used to characterize epitopes on proinsulin and insulin. In contrast to x-ray studies, the molecules can be used at low concentrations in soluble form. In spite of the technical differences, our data are in accordance with the confirmation of proinsulin suggested by x-ray crystallography.[13]

MABs offer the possibility to define insulin and proinsulin structures at physiologic concentrations. They may be useful for screening atypical insulins in the serum of diabetic patients and can be used for further refinement of the measurement of insulin and proinsulin in plasma.

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