Monoclonal antibodies reacting with multiple epitopes on the human insulin receptor

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Monoclonal antibodies for the human insulin receptor were produced following immunization of mice with IM-9 lymphocytes and/or purified placental receptor. Four separate fusions yielded 28 antibodies, all of which reacted with receptor from human placenta, liver and IM-9 cells. Some antibodies cross-reacted to varying degrees with receptor from rabbit, cow, pig and sheep, but none reacted with rat receptor. At least 10 distinct epitopes were recognized as indicated by species specificity and binding competition experiments. All of these epitopes appeared to be on extracellular domains of the receptor as shown by binding of antibodies to intact cells. In some cases the epitopes were further localized to α or β subunits by immunoblotting. Several antibodies inhibited binding of ^{125}I -insulin to the receptor, some had no effect on binding, and others enhanced the binding of ^{125}I -insulin. It is concluded that these antibodies will be valuable probes of receptor structure and function.

INTRODUCTION

Antibodies have proved to be valuable reagents in studies of the structure, function and biosynthesis of a variety of cell surface receptors and other minor membrane proteins. Polyclonal antibodies for the insulin receptor have been obtained by immunization of rabbits with purified receptor (Jacobs & Cuatrecasas, 1981) or from human subjects with a rare syndrome in which autoantibodies for the receptor occur spontaneously (Kahn *et al.*, 1981). Such antibodies have contributed greatly to studies on the structure (Harrison & Itin, 1980; Jacobs *et al.*, 1980), function (Kasuga *et al.*, 1982; Gazzano *et al.*, 1983) and biosynthesis (Deutsch *et al.*, 1983; Hedo *et al.*, 1983) of the insulin receptor. At the functional level the value of such antibodies is limited by their polyclonal and therefore heterogeneous nature, resulting in the simultaneous recognition of multiple epitopes and a variety of different biological effects (Kahn *et al.*, 1977; Jacobs *et al.*, 1978).

Many aspects of insulin action and details of receptor structure remain unclear at the molecular level. In particular, the nature of the signal generated by hormone–receptor interaction, which mediates effects on intracellular enzymes, remains to be established (Jacobs & Cuatrecasas, 1983; Czech, 1985). A complete description will require identification and characterization of the different structural and functional domains of the receptor, to complement the recently published primary sequence (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). This information may also clarify structural and functional relationships with receptors for other growth factors.

Monoclonal antibodies, because of their fine specificity and availability in quantity in purified form, have been especially useful in providing new insights into the structure and function of receptors for acetylcholine

(Lindstrom *et al.*, 1983; Fuchs *et al.*, 1984) and epidermal growth factor (Schlessinger *et al.*, 1984; Parker *et al.*, 1984; Gill *et al.*, 1984). In these instances, substantial numbers of antibodies reacting with distinct sites, and with different biochemical effects, have been obtained. Monoclonal antibodies for the insulin receptor have been more elusive and very few have so far been described in detail (Roth *et al.*, 1982; Kull *et al.*, 1983). We have prepared 28 monoclonal antibodies which react with at least 10 epitopes on the human insulin receptor, and here describe the initial characterization of these antibodies. Preliminary accounts of some of these findings and other properties of the antibodies have been presented elsewhere (Soos & Siddle, 1985; Soos *et al.*, 1985, 1986).

EXPERIMENTAL

Materials

Bovine insulin was from Sigma (London) Chemical Co., Poole, Dorset, U.K. and highly purified, des-amido free, bovine insulin was a gift from Dr. D. Brandenburg. CNBr-activated Sepharose 4B was from Pharmacia, Milton Keynes, Bucks, U.K. Rabbit antisera for mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ and IgM and wheatgerm agglutinin–Sepharose were from Miles Biochemicals, Slough, Berks, U.K. Affinity-purified goat anti-(mouse immunoglobulin) antibody coupled to horseradish peroxidase was from Tago, Burlingame, CA 94010, U.S.A. Cellulose acetate filters (Nuflow, 0.22 μm) were from Oxoid, Basingstoke, Hants, U.K., and nitrocellulose paper (0.2 μm) was from Schleicher & Schull, Dassel, Germany. Na¹²⁵I (IMS 30) was from Amersham International, Amersham, Bucks, U.K. Poly(ethylene glycol) 6000 was from BDH Chemicals, Dagenham, Essex, U.K. Myeloma cell lines P3/NSI/1-Ag4-1 (NSI)

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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and NSO/1 (NSO) were a gift from Dr. C. Milstein. Mice were from Bantin & Kingman, Hull, Humberside, U.K. IM-9 lymphocytes were obtained initially from Flow Laboratories, Irvine, Scotland.

Sheep anti-(mouse immunoglobulin) and sheep anti-(rabbit immunoglobulin) antibodies were prepared as described previously (Soos & Siddle, 1982), and coupled to aminocellulose to obtain immunoabsorbents also as described previously (Hales & Woodhead, 1980). Insulin-Sepharose was prepared by coupling bovine insulin to Sepharose 4B via a spacer arm as described by Fujita-Yamaguchi *et al.* (1983).

Normal human placenta was freshly obtained at delivery. Human liver was obtained at post mortem approx. 24 h after death. Animal livers were obtained immediately after killing from a slaughterhouse.

Radioiodinations

Purified human placental insulin receptor (Fujita-Yamaguchi *et al.*, 1983) was iodinated using a stoichiometric chloramine-T method (Roth, 1975). Na^{125}I (0.1–0.2 mCi in 25 μl of 0.5 M-sodium phosphate, pH 7.4) was added at room temperature to receptor (1–2 μg in 100 μl of 0.05 M-Tris/HCl, pH 7.4, containing 0.1 mM-PMSF and approx. 0.5% Triton X-100), followed by the addition, in 10 μl portions over 20–25 min, of 50 μl of chloramine-T (20 $\mu\text{g}/\text{ml}$ in 0.05 M-sodium phosphate buffer, pH 7.4). Iodinated receptor was separated from free iodide and Triton micelles by applying the reaction mixture to an Ultrogel AcA-34 column (0.9 cm $\times$ 58 cm) at 4 °C and eluting with 0.05 M-Tris/HCl (pH 7.4)/0.1 mM-PMSF/0.1% Triton X-100, with a flow rate of 2 ml/h. Fractions (0.5 ml) were collected and those containing receptor were pooled. Approx. 2–3% of the total radioactivity was incorporated into protein, giving a specific radioactivity of approx. 3–5 $\mu\text{Ci}/\mu\text{g}$.

Mono- ^{125}I -insulin with a specific radioactivity of 150–200 $\mu\text{Ci}/\mu\text{g}$ was prepared from highly purified bovine insulin as described by Linde *et al.* (1981). Sheep anti-(rabbit immunoglobulin) and sheep anti-(mouse immunoglobulin) antibodies were affinity-purified and iodinated as described previously (Hales & Woodhead, 1980).

Assays

Immunoprecipitation of ^{125}I -receptor. Antibody was incubated with ^{125}I -receptor (2000 c.p.m.) in 100 μl of Buffer A (0.075 M-Tris/HCl, pH 7.3 at room temperature, containing 0.03 M-NaCl, 0.01 M-glucose, 0.5 mM-EDTA, 0.1 mM-PMSF and 0.1% BSA) containing 0.05% Triton X-100 for 2 h at 20 °C or 18 h at 4 °C. Sheep anti-(mouse immunoglobulin) adsorbent (0.25 mg in 50 μl of the same buffer) was then added for 30 min at 20 °C or 2 h at 4 °C. Incubations were diluted with 1 ml of cold, 0.1 M-sodium phosphate buffer, pH 7.4, containing 1% BSA and 0.1% Triton X-100, centrifuged at 2000 g for 10 min at 4 °C and the pellets washed once with the same buffer, before counting for radioactivity.

Immunoprecipitation of receptor- ^{125}I -insulin complexes. Receptor, solubilized from placental membranes with 2% (v/v) Triton X-100 (Fujita-Yamaguchi *et al.*, 1983), was preincubated for 18 h at 4 °C with ^{125}I -insulin (20000 c.p.m., 100 pM) in 50 μl of Buffer A containing 0.05% Triton X-100. Receptor-bound ^{125}I -insulin was

determined by precipitation with poly(ethylene glycol) 6000 as previously described (Baron & Sonksen, 1982). Sufficient receptor was used to bind 5–10% of the ^{125}I -insulin under these conditions. Antibody (50 μl) was then added for a further 18 h at 4 °C and antibody-bound radioactivity was determined using a sheep anti-(mouse immunoglobulin) adsorbent as described above.

Insulin binding to membranes. Placental membranes (Fujita-Yamaguchi *et al.*, 1983) in 100 μl of buffer A were preincubated with 100 μl of antibody or insulin for 2 h at 4 °C, before addition of ^{125}I -insulin (20000 c.p.m., 100 pM) in 50 μl of buffer A for a further 18 h at 4 °C. Membrane-bound radioactivity was separated by filtration through 0.22 μm cellulose acetate filters, presoaked in 0.1 M-sodium phosphate buffer (pH 7.4)/1% BSA, followed by washing with 10 ml of the same ice-cold buffer. Sufficient membranes were used to bind 5–10% of the added radioactivity in the absence of antibody or insulin. Non-specific binding was determined by incubation with 1 μM -insulin.

Insulin binding to IM-9 cells. ^{125}I -insulin binding to IM-9 lymphocytes was determined as described by Roth *et al.* (1982) by preincubating 100 μl of cells for 30 min at 15 °C with 100 μl of antibody or insulin, before addition of 50 μl of ^{125}I -insulin for a further 75 min at 15 °C.

Production of monoclonal antibodies

The monoclonal antibodies described were obtained from Balb/c mice following two different immunization regimes. One group (92 mice including those coded numbers 47 and 83) was given two intraperitoneal injections of IM-9 lymphocytes [(2–4) $\times 10^6$ cells in saline] at 1 month intervals and then rested for 5–6 months. The second group (28 mice including those coded numbers 18 and 25) received two to five subcutaneous and footpad injections of purified receptor (0.5–2 μg in Freund's adjuvant) over several months, the receptor being obtained at this time by the method of Jacobs & Cuatrecasas (1981). Mice were test bled from the tail 10–15 days after boosting, and sera were monitored for the presence of anti-receptor antibody as described above. Six mice from each group with the highest serum antibody titres then received an intravenous boost, 4 days before fusion, with 1–2 μg of purified receptor prepared now by the improved method of Fujita-Yamaguchi *et al.* (1983). Spleen cells from these mice were fused with NSI (mice 25, 47, 83) or NSO (mouse 18) myeloma cells by using standard techniques (Galfre & Milstein, 1981). The cells from each fusion were plated onto 384 wells of microtitre trays, and vigorous hybrid growth was normally obtained in all wells. Culture supernatants were screened for anti-receptor antibodies by immunoprecipitation of ^{125}I -receptor as described above. Positive wells were detected in only five out of the 12 fusions, giving 36 persistent positive wells in all. These cells were cloned twice at limiting dilution, using spleen cell feeder layers. Cloning was successful for 28 cell lines which were then grown as ascitic tumours in mice. Antibody for characterization was partially purified from ascites fluid by precipitation with 40% saturated (v/v) $(\text{NH}_4)_2\text{SO}_4$ followed by reconstitution in PBS to the original ascites volume.

Isotyping of antibodies

Chain class was determined in the ^{125}I -receptor binding assay by using immunoadsorbents containing rabbit antibodies specific for mouse immunoglobulin subclasses, prepared as described by Soos & Siddle (1982). Results were confirmed in a dot immunobinding assay (Beyer, 1984).

Tissue and species specificity of antibodies

Crude microsomal membranes and wheatgerm agglutinin-Sepharose purified receptors were prepared from human tissues and animal livers as described by Fujita-Yamaguchi *et al.* (1983) for placenta. The specificity of antibodies was determined by testing their ability to inhibit ^{125}I -insulin binding to membranes or by their ability to immunoprecipitate receptor- ^{125}I -insulin complexes as described above.

Investigation of antigenic determinants on the insulin receptor

The relationship between the binding sites for different antibodies was investigated by competition studies, in which the ability of soluble antibody to inhibit binding of ^{125}I -antigen to an antibody on solid phase was tested. Antibodies were bound to solid phase by incubating 10 mg of sheep anti-(mouse immunoglobulin) adsorbent with anti-receptor antibody (1:8 dilution) in 2.5 ml of 0.05 M-Tris/HCl (pH 7.4)/0.1% Triton X-100/0.1 mM-PMSF for 18 h at 4 °C, followed by extensive washing with the same buffer. ^{125}I -receptor (5000 c.p.m.) was preincubated with soluble antibody (2 μl) and non-immune mouse serum (3%, v/v) in 100 μl of 0.05 M-Tris/HCl (pH 7.4)/0.1% Triton X-100 for 18 h at 4 °C, before addition of 0.25 mg of adsorbent containing mouse anti-receptor antibody (100 μl) for 30 min at 20 °C. Incubations were then terminated by the addition of 2 ml of cold 0.1 M-sodium phosphate (pH 7.4)/0.1% BSA/0.1% Triton X-100, and radioactivity bound to solid phase anti-receptor antibody was determined after centrifugation at 2000 *g* for 10 min at 4 °C.

Immunoblotting

Purified placental insulin receptor (approx. 10–20 μg) was reduced in sample buffer containing 50 mM-dithiothreitol, and electrophoresed across the whole width (15 cm) of a 7.5% polyacrylamide gel (Laemmli, 1970). The gel was blotted onto 0.2 μm nitrocellulose paper as described previously (Burnette, 1981) and the paper was then incubated in PBS containing 2% (w/v) Marvel dried milk for 1 h at 20 °C. Strips of this paper were incubated with antibody at a dilution relative to ascites fluid of 1:10, in 3 ml of PBS containing 2% Marvel and 0.5% Tween 20, for 2 h at 20 °C. The strips were washed in PBS/2% Marvel/0.5% Tween, PBS/0.5% Tween, PBS/0.05% SDS/0.5% Triton X-100/0.5% sodium deoxycholate/2.5 mM EDTA, and finally PBS/0.05% Tween. They were then incubated with horseradish peroxidase-conjugated goat anti-(mouse immunoglobulin) antibody at a dilution of 1:2000 in 3 ml of PBS/2% Marvel/0.5% Tween for 2 h at 20 °C. Washing was carried out as previously and then with PBS alone, before addition of peroxidase substrate consisting of diaminobenzidine (0.2 mg/ml), CoCl_2 (0.3 mg/ml) and H_2O_2 (0.06 mg/ml) in PBS.

RESULTS

Production and preliminary characterization of antibodies

Only 12 mice, out of 120 immunized, developed a serum titre of anti-receptor antibody which was considered sufficient to justify a fusion (at least half-maximal response in one or more screening assays with 1:200 dilution of serum). Most of the monoclonal antibodies obtained came from two animals (numbers 18 and 83) which had easily the highest serum titres. There were also important qualitative differences between the serum antibodies in different animals. Of those immunized initially with IM-9 cells, mouse 47 produced predominantly antibody which inhibited insulin binding and mouse 83 predominantly non-inhibitory antibody, while other mice had both types of antibody. It was not possible to assess the serum antibody in this way for mice immunized with affinity-purified receptor (including numbers 18 and 25) because of the presence in these sera of anti-insulin antibodies.

Twenty-eight different monoclonal antibodies were obtained, from four separate fusions (Table 1). These were detected initially in the screening assay with ^{125}I -receptor, and were then studied further to determine their effect on ^{125}I -insulin binding to IM-9 lymphocytes and their ability to precipitate receptor-bound ^{125}I -insulin (Fig. 1). The antibodies fell into four major groups on this basis. All three antibodies from mouse 47 strongly inhibited ^{125}I -insulin binding, and did not bind the receptor- ^{125}I -insulin complex. A large group of 15 antibodies from mouse 18 had relatively little effect in the binding inhibition assay but reacted well with the receptor- ^{125}I -insulin complex. Three antibodies from mice 25 and 83 reacted well in both assays. Seven antibodies from mice 18 and 83 reproducibly increased the binding of ^{125}I -insulin to IM-9 cells, in addition to binding the receptor- ^{125}I -insulin complex.

The antibodies 25-49, 47-9, 47-46 and 83-14 which most strongly inhibited ^{125}I -insulin binding to IM-9 cells were investigated further to determine the concentration-dependence of this effect. Half-maximal inhibition was obtained at final antibody dilutions of 1:25000–1:75000 relative to ascites fluid, or approx. 0.2–1 nM assuming a typical concentration of 2–5 mg/ml in neat tumour fluid. The antibodies were therefore approximately equipotent with insulin itself, which was half-maximally effective at 0.5 nM under the same conditions.

Most antibodies were of IgG₁ subclass, although some IgG_{2a}, IgG_{2b} and IgM antibodies were also obtained (Table 1).

Tissue specificity

The reaction of antibodies with receptor from human placenta, human liver and IM-9 cells was studied in ^{125}I -insulin binding-inhibition or co-precipitation assays as appropriate. Typical results are shown in Figs. 2 and 3. The antibodies reacted well with all tissues although there were minor reproducible differences. Thus, the inhibitory antibodies were consistently less effective with liver than with placental or IM-9 membranes. This was evident both in a rightward shift and shallower slope of the dose-response curve, and in failure completely to inhibit specific binding of ^{125}I -insulin at the highest concentration tested (Fig. 2). Some, but not all, of the non-inhibitory antibodies similarly were somewhat less effective in precipitating ^{125}I -insulin bound to solubilized

Table 1. Summary of antibody properties

Antibody*	Isotype	Reaction in screening assays		Species cross-reaction			Epitope	
		IM-9 binding†	Co-precipitation‡	Rabbit§	Bovine§	Pattern	Immunoblot¶	Code**
47-6	M	—	—	±	+	(4b)	0	1A
47-9	G1	—	—	+	++	4b	α	1B
47-46	G1	—	—	+	++	4b	α	
25-49	G1	—	+	+	—	4c	α	2
83-14	G2a	—	+	—	(+)	5e	α	3
83-22	G1	—	+	—	(±)	5e	α	
18-26	G1	0	+	+	(+)	5c	α	4
18-34	G2a	0	+	—	(±)	5f	α?	5
18-43	G2a	0	+	±	(±)	5d/f		6
18-23	G1	0	+	+	(±)	5c	β	7A
18-24	G1	0	+	+	(±)	5c		
18-38	G1	0	+	+	(+)	5c		
18-39	G1	0	+	+	(+)	5c		
18-41	G1	0	+	±	(±)	5c/d		
18-36	G1	0	+	(±)	(±)	5d	β	7B
18-44	G2b	0	+	(±)	(±)	5d	β	
18-28	G1	0	+	—	(+)	5f	0	7C
18-37	G1	0	+	—	(±)	5f		
18-42	G2b	0	+	—	(±)	5f		
18-40	G1	0	+	+	+	5b	β	7D
18-45	G1	0	+	+	+	5b/c		
83-7	G1	+	+	++	++	5a	0	8A
83-16	G1	+	+	++	++	5a	0	
83-12	G1	+	+	++	++	5a	0	8B
83-15	G1	+	+	++	++	5a		
83-19	G1	+	+	++	++	5a		
18-29	G1	+	+	+	±	5c	α?	9
18-146	G1	+	+	—	±	5f	0	10

* The code numbers identifying individual antibodies are preceded by the number of the mouse from which they originate.

† Reactivity of antibodies (at 1:100–1:1000 dilution) in the assay of ¹²⁵I-insulin binding to IM-9 cells is classified as greater than 80% inhibition (—), 0–50% inhibition (0) or enhancement of binding (+) as shown in Fig. 1.

‡ Reactivity of antibodies (at 1:100–1:1000 dilution) in the assay of ¹²⁵I-insulin–receptor co-precipitation is classified as immunoprecipitation of all (+) or none (—) of poly(ethylene glycol)-precipitable radioactivity.

§ Cross reaction is defined from relative antibody concentrations reacting with rabbit or bovine liver receptor compared with human liver. Where possible titres were compared at half-maximal binding levels, the relative cross-reactions being 50–100% (++), 1–10% (+), 0.1–1% (±) or insignificant reaction (—). Results in parentheses indicate titration curves which were not parallel to those with human liver.

|| The pattern of cross reaction is indicated by the Figure number which it most closely resembles.

¶ Subunit reactivity (α or β) in immunoblots with purified receptor (Fig. 7) is indicated. Lack of reaction is denoted by 0, and gaps indicate antibodies which were not tested.

** Antibodies were grouped into epitopes, on the basis of differences in species specificity and/or binding competition (Fig. 6). The numbered epitopes which were clearly distinct (1–10) were in some cases subdivided (A–D) where antibodies showed relatively minor differences but also significant similarities in properties.

liver rather than placental receptors, both in terms of concentration dependence and maximal effect (Fig. 3).

Species specificity

Species specificity was investigated by using human, rabbit, bovine, porcine, sheep and rat liver membranes in both the ¹²⁵I-insulin binding-inhibition and co-precipitation assays. None of the antibodies reacted with rat liver receptors (results not shown). All antibodies reacted very similarly with bovine, porcine and sheep receptors, so that for simplicity only results with bovine liver are shown in each case.

The antibodies which inhibited insulin binding displayed three main patterns of cross-reaction. One group, typified by antibody 47-9, reacted equally with human

and bovine receptors and rather less well with rabbit, while antibody 25-49 showed no reaction with bovine receptors (Fig. 4). Antibody 83-14 on the other hand reacted with bovine but not rabbit receptors in the inhibition assay (result not shown), as in the co-precipitation assay (Fig. 5).

The antibodies studied in the co-precipitation assay displayed complex patterns of cross-reaction (Fig. 5). Two groups reacted well with all species except rat, exemplified by antibody 83-7, which bound equally to human, bovine and rabbit receptors, and antibody 18-40 which showed preference for human receptors. Two further groups, typified by antibodies 18-24 and 18-44, reacted weakly with both bovine and rabbit receptors, with consistent differences in the shape of the dose–

Fig. 1. Comparison of reactivity of antibodies in ^{125}I -insulin-receptor co-precipitation and ^{125}I -insulin binding inhibition assays

Assays were performed as described in the Experimental section, using solutions of antibodies at a dilution of 1:100 relative to ascites fluids. In the co-precipitation assays (open bars) results are expressed as a percentage of total receptor-bound radioactivity (2800 c.p.m.), determined by precipitation with poly(ethylene glycol) (PEG) before addition of antibody. (After incubation with antibody the precipitable radioactivity varied with different antibodies, but fell to approx 70% in controls as a result of dilution alone.) Non-immune antibodies of irrelevant specificity precipitated less than 1% of the poly(ethylene glycol)-precipitable radioactivity. In the binding inhibition assay (filled bars) results are expressed as a percentage of the specific cell-bound radioactivity measured in the absence of antibody. Non-immune mouse antibodies had little effect on binding, giving a value of $99 \pm 16\%$ (mean $\pm$ S.D., six determinations).

response curves. A final two groups, as shown for antibodies 83-14 and 18-28, showed moderate or low cross-reaction respectively with bovine receptor, and no reaction with rabbit receptor.

Analysis of epitopes

The effects of antibodies on insulin binding and the various patterns of species cross-reaction in themselves indicated recognition of several distinct sites on the receptor. These differences in epitope were further analysed by competitive binding experiments and by immunoblotting.

Competition studies were carried out by testing the effect of preincubation of ^{125}I -receptor with one antibody in solution on subsequent binding to a second immobilized antibody (Fig. 6). The observed inhibition was not always all or none as might be predicted on simple models of steric competition. Moreover, in a few cases, mutual reciprocal inhibition was not observed. For instance, soluble antibody 83-14 blocked binding to immobilized antibody 47-9, but not vice versa, and soluble antibody 83-7 blocked binding to immobilized antibody 18-38, but not vice versa. Nevertheless, similarities and differences in overall pattern enabled the antibodies to be placed in a variety of distinct epitope groups (Table 1).

Attempts were made to localise the epitopes to either the α or β subunit of the receptor by means of immunoblotting, after separation of the subunits on polyacrylamide gels under reducing conditions (Fig. 7). A polyclonal rabbit anti-receptor serum reacted strongly with both subunits. The reactions of the monoclonal antibodies were weaker but in several cases were unequivocally with one or other of the subunits (Table 1). Some antibodies, such as 83-7, 83-15 and 83-16, which appeared similar in reactivity by various criteria, showed no detectable reaction in immunoblotting experiments with reduced receptor.

Binding of monoclonal antibodies to intact IM-9 cells at 15°C was investigated in an indirect assay using ^{125}I -rabbit anti-(mouse immunoglobulin) antibody. Conditions for saturation of available binding sites for both first and second antibodies are difficult to achieve without also introducing high levels of 'non-specific' binding, and the extent of binding of labelled antibody may also depend on monoclonal antibody subclass. Such an assay does not therefore easily provide a quantitative measure of antigen concentration. Nevertheless, all antibodies showed significant binding to IM-9 cells, within a 2.5-fold range of radiolabelled second antibody bound (results not shown), indicating that all epitopes were accessible at the cell surface and present in broadly similar numbers.

DISCUSSION

The aim of the work described in this paper was to obtain antibodies reacting with multiple sites on the insulin receptor, for use as probes of receptor structure and function; 28 antibodies, recognizing at least 10 epitopes, were obtained from four fusions. Different animals yielded distinct groups of antibodies, indicating considerable individual variation in the epitopes recognized. It is possible that the form of immunogen used, either IM-9 cells or purified receptor, has some influence on the dominant epitopes expressed. The screening assay employed may also introduce bias in the type of antibody obtained, either inhibitory or non-inhibitory with respect to insulin binding (Roth *et al.*, 1982; Kull *et al.*, 1983), although we deliberately used an assay which detected antibodies of both types. Recently, Morgan & Roth (1986) described a large number of monoclonal antibodies

Fig. 2. Tissue specificity of antibodies which inhibit insulin binding

Binding inhibition assays were carried out by preincubating membranes from human placenta (●) liver (▲) or IM-9 lymphocytes (▽) with solutions of insulin (a), antibody 47-9 (b) or antibody 25-49 (c) at the concentrations indicated before addition of ^{125}I -insulin. Results are expressed as a percentage of specific membrane-bound radioactivity measured in the absence of antibody or unlabelled insulin (1000–1200 c.p.m.) Non-specific binding was 100 c.p.m.

Fig. 3. Tissue specificity of antibodies reacting in the ^{125}I -insulin-receptor co-precipitation assay

Co-precipitation assays were carried out by preincubating wheatgerm agglutinin-Sepharose-purified receptors from human placenta (●) or liver (▲) with ^{125}I -insulin (20000 c.p.m.) before addition of various concentrations of antibodies 83-7 (a), 18-28 or 83-14 (c). Results are expressed as a percentage of receptor-bound radioactivity (liver, 1500 c.p.m.; placenta, 2800 c.p.m.) determined by precipitation with poly(ethylene glycol) (PEG) at the time of addition of antibody.

Fig. 4. Species specificity of antibodies that inhibit insulin binding

Binding inhibition assays were carried out by preincubating membranes from human (▲), bovine (△) or rabbit (○) liver with solutions of insulin (a), antibody 47-9 (b) or antibody 25-49 (c) at the concentrations indicated. Results are expressed as a percentage of specific membrane-bound radioactivity measured in the absence of antibody or unlabelled insulin (1000–2000 c.p.m.).

Fig. 5. Species specificity of antibodies reacting in the ^{125}I -insulin-receptor co-precipitation assay

Co-precipitation assays were carried out by preincubating wheatgerm agglutinin-Sepharose-purified receptors from human (▲), bovine (△) or rabbit (○) liver with ^{125}I -insulin (20000 c.p.m.) before addition of various concentrations of antibodies 83-7 (a), 18-40 (b), 18-24 (c), 18-44 (d), 18-14 (e) or 18-28 (f). Results are expressed as a percentage of receptor-bound radioactivity (human, 1500 c.p.m.; bovine, 1800 c.p.m.; rabbit, 2500 c.p.m.) determined by precipitation with poly(ethylene glycol) at the time of addition of antibody.

for epitopes on the intracellular protein kinase domain of the insulin receptor, and concluded that this represents the major antigenic region of the receptor. However, most of these antibodies came from a single animal. All our antibodies in contrast appeared to react with extracellular domains as shown by binding to intact IM-9 cells. Our failure to obtain any antibodies similar to those of Morgan & Roth (1986) may have resulted from the use in screening assays of receptor which was partly proteolysed (Fujita-Yamaguchi, 1984) and therefore probably deficient in protein kinase domain.

Several antibodies were identified which inhibited insulin binding, involving at least three distinct epitopes, and all these antibodies were shown to react with the α subunit of the receptor on immunoblots (Table 1). None of the antibodies reacting with β -subunit inhibited binding, and some α -subunit reactive antibodies (e.g. 18-26) were also non-inhibitory. These observations are consistent with the demonstration by photoaffinity-labelling and cross-linking experiments (Pilch & Czech, 1979; Wisher *et al.*, 1980) that the binding site for insulin is predominantly associated with the α -subunit. Some antibodies (e.g. 83-14 and 25-49) were effective both at inhibiting ^{125}I -insulin binding and in the co-precipitation of receptor with bound ^{125}I -insulin. Thus although antibody blocked binding of insulin to receptor, insulin did not necessarily reciprocally block the binding of antibody. However, in the former experiment antibody was preincubated in excess with the receptor, whereas in the latter ^{125}I -insulin was present only in tracer quantities occupying a

small fraction of potentially available binding sites. What does then require explanation is why some inhibitory antibodies (e.g. 47-9 and 47-46) were unable to precipitate receptor- ^{125}I -insulin complexes. This is in part accounted for by the finding that these antibodies accelerate the dissociation of ^{125}I -insulin from receptor in a manner similar to high concentrations of insulin (M. A. Soos & K. Siddle, unpublished work). This suggests that these antibodies induce or stabilize conformational changes similar to those responsible for negative co-operativity of insulin binding (De Meyts *et al.*, 1976). Some other antibodies in contrast enhanced the binding of ^{125}I -insulin to IM-9 cells and solubilized receptor (Fig. 1), as previously observed with a polyclonal antiserum (Grunfeld, 1984). These findings indicate that there are multiple conformational states of the receptor which may be favoured by the binding of different antibodies. Conformational flexibility must also be invoked to account for insulin-induced transmembrane activation of receptor kinase activity (Kasuga *et al.*, 1982) and changes in sensitivity of the receptor to proteolysis (Pilch & Czech, 1980). The phenomenon of negative co-operativity (De Meyts *et al.*, 1976) further suggests that such conformational changes are transmitted between ligand binding sites on adjacent receptor subunits.

These conformational considerations complicate the interpretation of experiments to investigate competition between different antibodies in binding to receptor (Fig. 6), which were nevertheless valuable in identifying groups of antibodies recognizing distinct epitopes. The simplest

Fig. 6. Epitope analysis by binding competition

Results are expressed as the percentage inhibition of binding of ^{125}I -receptor to representative immobilized antibodies following preincubation of receptor with the same (hatched bars) or different (open bars) antibodies in solution. The scales at the side indicate 0 (bottom) and 100% (top) inhibition for each antibody.

explanation for such competition is in terms of steric hindrance, although lack of reciprocal inhibition with some pairs of antibodies implicates additional factors. Given two-fold symmetry of the receptor (Czech, 1985) it is possible to envisage a disposition of epitopes which would lead to non-reciprocal inhibition on the basis of steric hindrance alone. Receptor heterogeneity arising from partial proteolysis (Fujita-Yamaguchi, 1984) and resulting in selective loss of epitopes would further complicate the analysis. Alternatively or additionally the conformation of some epitopes may be sensitive to the binding of antibodies at other sites. Some epitopes are very dependent on conformation, as indicated by failure of antibodies of the 83-7 and 83-15 groups to react with receptor on immunoblots.

The present results do not establish unequivocally that all the antibodies obtained are monospecific for the insulin receptor. The degree of cross-reaction with IGF

receptors for instance (Roth *et al.*, 1983; Kull *et al.*, 1983) remains to be established. In the case of epidermal growth factor receptors some monoclonal antibodies reacted with carbohydrate determinants also found on other cell surface molecules (Parker *et al.*, 1984). However, several lines of evidence point to a high degree of specificity in the antibodies we have described. One antibody (18-42) has been used in the immunoaffinity purification of placental insulin receptor (O'Brien *et al.*, 1986) and another (83-14) in the specific immunoprecipitation of receptor from biosynthetically labelled lymphocytes (J. Bellatin, E. S. Lennox & K. Siddle, unpublished work). In both cases, analysis of the immunoreactive material by SDS/polyacrylamide-gel electrophoresis revealed the α and β subunits of receptor as the major polypeptides. The binding assays we have carried out also showed that all antibodies bound to intact IM-9 cells to a similar extent irrespective of individual epitopes, suggesting that the major reaction is with the same macromolecule. However, binding studies by Scatchard analysis with monovalent antibody fragments will be necessary to quantify the precise number of sites for each antibody together with the respective binding affinities. In terms of ability to inhibit binding of ^{125}I -insulin, antibodies 25-49 and 47-9 appeared to be of similar potency to insulin itself. Some antibodies however were clearly of low avidity and it was one of these which proved useful in the immunoaffinity purification of insulin receptors (O'Brien *et al.*, 1986).

Our findings to date provide no clear support for the existence of structural heterogeneity of insulin receptors within or between the tissues studied (Figs. 2 and 3), although they do not rule out the possibility of different affinity states *in situ* within a given population of receptors. Some small differences were observed in the reactivity of antibodies with receptor from liver compared with placenta or IM-9 cells. The possibility exists that these result from post-mortem changes in the liver before processing rather than inherent tissue differences, and further work is required to investigate this. We saw no difference in reactivity of any of our antibodies with IM-9 cells compared to placenta, although a previous report described a monoclonal antibody which was somewhat more effective at inhibiting insulin binding to IM-9 cells than to placenta or adipocytes (Roth *et al.*, 1982). This antibody also shows discrimination in the case of receptors from human brain, with which it reacts weakly relative to placenta (Roth *et al.* 1986). It is possible therefore that there are subtle differences between receptors in different tissues, perhaps reflecting post-translational modifications.

The present large group of antibodies reveal a considerable degree of species divergence in receptor structure even in regions close to the insulin binding site (Figs. 4 and 5). This contrasts with the previously assumed conservation of structure during evolution, based on relative binding affinity of different insulins (Kemmler *et al.*, 1978). Substantial homology between species was also indicated by the cross-reaction of human autoantibodies with rat receptor (Kahn *et al.*, 1977) and of rabbit antisera to rat receptor with human tissues (Kull *et al.*, 1982). None of our monoclonal antibodies for the human receptor reacted with rat receptor, as reported also for other monoclonal antibodies (Roth *et al.*, 1982; Kull *et al.*, 1983). This possibly reflects a selection against any common epitopes which (assuming rat and mouse

Fig. 7. Epitope analysis by immunoblotting

Protein blots of purified receptor, which had been fractionated by SDS/polyacrylamide-gel electrophoresis, were probed with monoclonal antibodies (1:10 dilution relative to ascites) or a rabbit polyclonal antiserum (1:100 dilution of serum), as described in the Experimental section. Mobilities of standard molecular mass markers are indicated at the side in kDa. Antibody codes are indicated above each strip.

receptors to be very similar) might not be recognized as foreign in rodents. On the other hand, mouse monoclonal antibodies for the intracellular protein kinase domain of human insulin receptor do cross-react with rat receptor (Morgan & Roth, 1986), indicating that this structure at least is highly conserved and yet still immunogenic under suitable conditions.

Preliminary studies indicate that several of our antibodies exert insulin-like effects on the metabolism of isolated human adipocytes (Soos *et al.*, 1986) and it will be interesting to characterize the binding sites and biochemical effects of these antibodies more fully in relation to current theories of the mechanism of insulin action.

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