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Effects of anti-insulin antibody on insulin binding to liver membranes: evidence against antibody-induced enhancement of insulin binding to the insulin receptor

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Summary. In the presence of anti-insulin antibody, 2- to 3-fold enhancement of ^{125}I-insulin binding to liver membranes was observed when binding was estimated by the radioactivity of ^{125}I-insulin bound to the membrane pellets. However, after ^{125}I-insulin was covalently cross-linked to liver membranes using disuccinimidyl suberate in the presence of anti-insulin antibody, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography showed that ^{125}I-insulin bound to the α-subunit of the insulin receptor was inhibited by anti-insulin antibody in an dose-dependent manner. More importantly, at an anti-insulin antibody dilution range between 1:50 and 1:5,000, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed two ^{125}I-labelled bands of mol wt 62,000 and 27,000, while only one band of mol wt 130,000 was revealed in the absence of anti-insulin antibody. These Mr = 62,000 and Mr = 27,000 bands were found to be the heavy and the light chain of anti-insulin IgG molecules respectively. Pepsin digested anti-insulin serum had only an inhibitory effect on ^{125}I-insulin binding to liver membranes. Non-immunized guinea pig serum or IgG completely abolished the enhanced effect of anti-insulin antibody. Further, this enhanced effect was inhibited by Fc fragment-specific anti-IgG serum or H&L-chain-specific anti-IgG serum in a dose-dependent manner. Protein A also inhibited the effect of anti-insulin antibody. In IM-9 lymphocytes and human red blood cell ghosts, which have no Fcγ receptors, enhancement of insulin binding was not observed in the presence of anti-insulin antibody. These data suggest that anti-insulin antibody-induced enhancement of insulin binding to liver membranes is not due to the enhanced binding to the insulin receptor itself but probably due to the binding of insulin-anti-insulin antibody complex to the Fcγ receptor.

Key words: Anti-insulin antibody, insulin receptor, insulin binding, cross-linking, disuccinimidyl suberate, Fcγ receptor, liver membrane.

It is reported that anti-insulin antibody(AIAB) at low concentrations induced cross-linking or aggregation of insulin-receptor complex and enhances the biological activity of bound insulin [1]. Shechter et al. [2] demonstrated that AIAB at optimal concentrations markedly increased ^{125}I-insulin binding to liver membranes and 3T3-C2 fibroblasts but not to adypocyte membranes. They suggested that AIAB caused aggregation of the insulin-receptor complex on liver membranes and increased the affinity of the insulin receptor. They also suggested that the inability of AIAB to increase the insulin binding to adypocyte membranes was due to the differences in structural organization of the insulin receptor on adypocytes and liver membranes [3]. Recently, Lyen et al. [4] also reported AIAB-induced enhancement of insulin binding to liver membranes and demonstrated AIAB-induced aggregation of ferritin-insulin-occupied receptor using electron microscopy.

To investigate whether AIAB truly enhances the insulin binding to the insulin receptor itself, we used the methods of chemical cross-linking and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography [5].

Materials and methods

Materials

Monocomponent porcine insulin was purchased from Novo Inc., Copenhagen, Denmark. ^{125}I-porcine insulin (160–220 μCi/μg) was purchased from Dainabot Co., Ltd. Tokyo, Japan. Most of the iodine atoms were attached to the tyrosine in A 14 position and a small quantity of them were in A 19 position. Disuccinimidyl suberate, pepsin, and protein A were purchased from Sigma Chemical Co., St Louis, Mo, USA. H&L-chain-specific goat anti-guinea pig IgG serum and Fc fragment-specific anti-IgG serum were purchased from Cappel Laboratories Inc., Cochranville, Pa, USA. Anti-insulin antiserum was obtained by immunizing guinea pig with monocomponent porcine insulin.

Methods

Membrane and cell preparations. Liver plasma membranes were prepared from male Wister rats (200–250 g) by the two-phase polymer

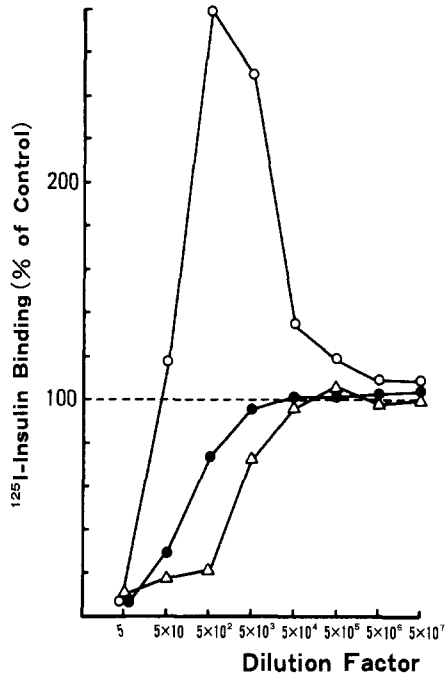

Fig. 1. The effects of anti-insulin antibody (AIAB) on ^{125}I-insulin binding to liver membranes (○), HRBC ghosts (△), and IM-9 cells (●). Liver membranes (100μg/ml), HRBC ghosts (2 mg/ml) or IM-9 cells (10^6/ml) were incubated with ^{125}I-insulin (0.2 ng/ml) in the presence of variously diluted AIAB. Results were expressed as a percent of control (100) in the presence of non-immunized guinea pig serum. Each point is the mean of triplicate determinations

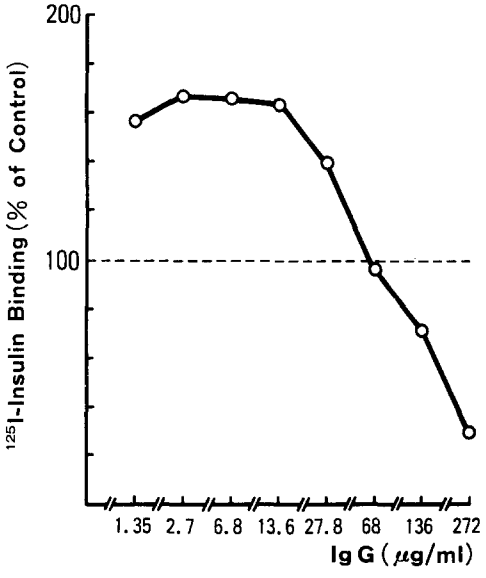

Fig. 2. The effect of purified IgG from anti-insulin antiserum on ^{125}I-insulin binding to liver membranes. Liver membranes (100 μg/ml) were incubated with ^{125}I-insulin (0.2 ng/ml) in the presence of various concentrations of anti-insulin IgG. Results were expressed as a percent of control (100) in the absence of anti-insulin IgG. Each point is the mean of triplicate determinations

method described by Lesko et al. [6]. Human red blood cell (HRBC) ghost membranes were prepared from a normal volunteer according to the method of Dodge et al. [7]. IM-9 lymphocytes were grown at 37 °C in RPMI 1640 medium containing 25 mmol/l Hepes base at pH 7.4 and 10% fetal calf serum. Cells in late log phase of growth were harvested by centrifugation at 600 *g* for 10 min before incubation.

Preparation of IgG. IgG was prepared from anti-insulin guinea pig serum or non-immunized guinea pig serum by ammonium sulfate precipitation and DEAE-cellulose column chromatography [8].

Pepsin digestion of anti-insulin serum. Anti-insulin guinea pig serum was directly digested with pepsin for 18 h at 37 °C according to the method described by Sire et al. [9]. Digested serum was dialysed against phosphate-buffered saline pH 7.4 for 48 h and finally concentrated to the initial volume by ultrafiltration membrane, Centriflo CF 25 (Amicon Co., Danvers, Mass, USA).

Insulin binding studies. Insulin binding to liver membranes, HRBC ghosts and IM-9 cells was conducted in 100 mmol/l Hepes buffer, ph 7.8, containing 10 mmol/l $MgCl_2$, 2 mmol/l EDTA, 10 mmol/l Glucose, 10 mmol/l $CaCl_2$, 50 mmol/l NaCl, 5 mmol/l KCl and 1% BSA. Insulin binding studies were performed for 24 h at 4 °C by incubating either liver membranes (100 μg/ml), HRBC ghosts (2 mg/ml) or IM-9 cells (10^6/ml) with ^{125}I-insulin (0.2 ng/ml) in the presence or absence of variously diluted AIAB.

In some experiments, purified IgG from anti-insulin serum was added. For experiments in which the effects of non-immunized guinea pig serum, IgG, anti-IgG sera or protein A were assessed, these materials were incubated with ^{125}I-insulin and AIAB simultaneously. Bound hormone was separated from unbound hormone by centrifugation (10,000 *g* for liver and HRBC ghost membranes and 600 *g* for IM-9 cells). Pellets were washed three times with assay buffer. Finally, ^{125}I-insulin binding was determined by the radioactivity in the pellets. Non-specific binding in the presence of 10^5 ng/ml insulin was determined and subtracted from total binding to yield specific binding. Non-specific binding was less than 20% of the total amount of bound hormone and was not significantly altered by AIAB. In these conditions, ^{125}I-insulin degradation was less than 5% of the total ^{125}I-insulin added and was not altered by AIAB as measured by the trichloroacetic acid precipitation method [10].

Cross-linking, gel electrophoresis and autoradiography. Liver membranes (400 μg/ml), HRBC ghosts (2 mg/ml) or IM-9 cells (10^6/ml) were incubated for 24 h at 4 °C with ^{125}I-insulin (10 ng/ml) in the presence or absence of various concentrations of AIAB. The incubation mixture was cooled to 0 °C and diluted 3-fold with assay buffer without BSA, and then centrifuged at 4 °C. After washing with the same buffer twice, the membranes or cell pellets were resuspended in the same buffer and freshly prepared disuccinimidyl suberate dissolved in dimethyl sulfoxide was added to a final concentration of 0.03 mmol/l [11]. After 15 min, the reaction was quenched by addition of 5 volumes of ice-cold 10 mmol/l Tris, 1 mmol/l EDTA, pH 7.4. This mixture was centrifuged and washed once, then boiled for 3 min in Laemmli's sample buffer [12] in the presence of 100 mmol/l dithiothreitol. ^{125}I-insulin was also covalently cross-linked to AIAB after incubation for 24 h at 4 °C without liver membranes and a portion of the mixture was added to the equal volume of twice concentrated sample buffer and then boiled. The discontinuous buffer system described by Laemmli [12] was used for analysis of the samples by SDS-PAGE. Separating gels contained 7.5% polyacrylamide. The gels were stained for 2 h in 0.025% Coomassie Blue R dissolved in 50% methanol, 10% acetic acid and were destained in 25% methanol, 7% acetic acid. The dried gels were autoradiographed with Fuji X-ray film (Fiji Photo Film Co., Ltd. Tokyo, Japan) for 24–72 h at −80 °C. As the standard of the mol wt, bovine plasma α_2-macroglobulin (reduced, 170,000), rabbit muscle phosphorylase b (97,400), bovine liver glutamate dehydrogenase (36,500), and soybean trypsin inhibitor (20,100) were used.

Results

The effects of various concentrations of AIAB on insulin binding to liver membranes, HRBC ghosts and IM-9 cells are shown in Figure 1. In liver membranes, ^{125}I-insulin binding was markedly inhibited by AIAB at a final

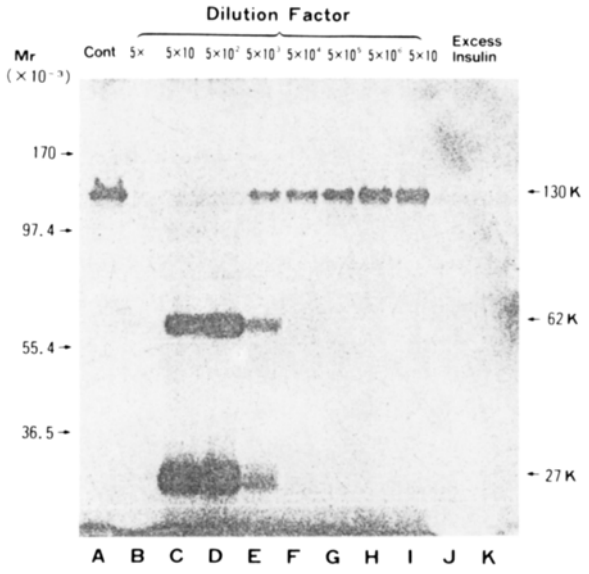

Fig. 3. Electrophoretic analysis of the effects of AIAB on ^{125}I-insulin binding to liver membranes. ^{125}I-insulin (10 ng/ml) and liver membranes (400 μg/ml) were incubated for 24 h at 4 °C in the absence (A) and the presence of variously diluted AIAB ranging from 1:5 to 1:5 × 10^7 (B to I). Excess amount of insulin (10^5 ng/ml) was added to the incubation mixture in the absence of AIAB (J), and in the presence of AIAB diluted by 1:500 (K). After incubation, cross-linking and SDS-PAGE followed by autoradiography were performed

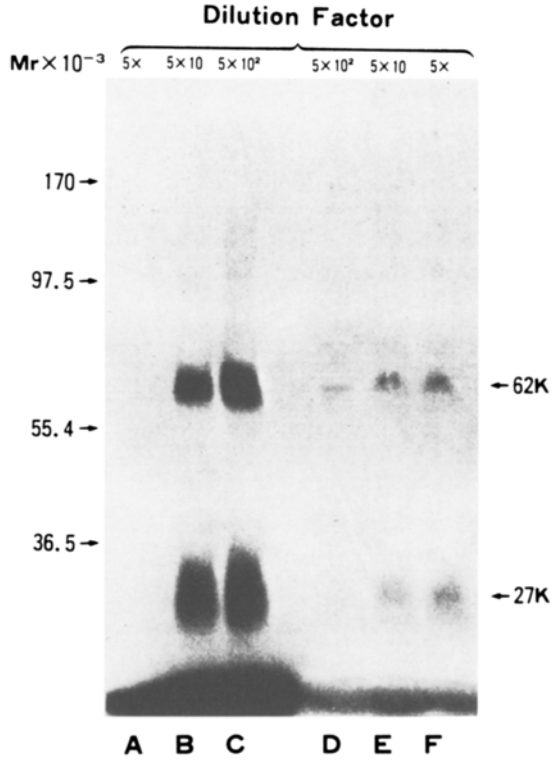

Fig. 4. Electrophoretic analysis of the effects of AIAB on ^{125}I-insulin binding to liver membranes. ^{125}I-insulin (10 ng/ml) and AIAB (dilution range from 1:5 to 1:500) were incubated for 24 h at 4 °C in the presence (A to C) and absence (D to E) of liver membranes. Cross-linking, SDS-PAGE and autoradiography were performed after incubation

dilution of 1:5, but about 3-fold stimulation was observed at an AIAB dilution of 1:500, followed by a return to the control level at lower concentrations. Enhancement of ^{125}I-insulin binding to liver membranes was also achieved by purified IgG from anti-insulin antiserum (Fig. 2). On the other hand, ^{125}I-insulin binding to HRBC ghosts and IM-9 cells was only inhibited by AIAB in a dose-dependent manner (Fig. 1).

When ^{125}I-insulin was covalently cross-linked to liver membranes by disuccinimidyl suberate, SDS-PAGE under reducing conditions revealed only one radioactive band of mol wt 130,000 in the presence of non-immunized guinea pig serum (Fig. 3, A). To assess the specificity of this band, ^{125}I-insulin was cross-linked in the presence of exess amount of insulin. Insulin abolished the Mr = 130,000 band (Fig. 3, J), suggesting that this band corresponded to the α-subunit of the insulin receptor [13, 14].

When ^{125}I-insulin was cross-linked to liver membranes in the presence of various dilutions of AIAB, the Mr = 130,000 band was abolished in a dose-dependent manner (Fig. 3, B-I). More importantly, SDS-PAGE revealed two major bands of mol wt 62,000 and 27,000 at an AIAB dilution range between 1:50 and 1:5,000 (Fig. 3, C-E). These two bands were also abolished in the presence of exess amounts of insulin (Fig. 3, K). In HRBC ghosts or IM-9 cells, SDS-PAGE and radioautography showed that the Mr = 130,000 band was abolished by AIAB in a dose-dependent manner but no other bands were visible (data not shown).

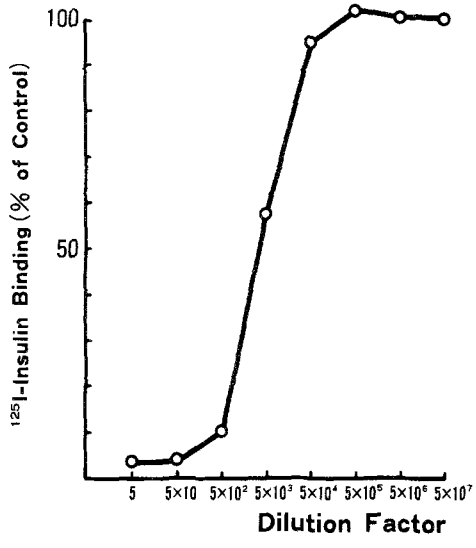

Fig. 5. The effect of pepsin-digested anti-insulin serum on ^{125}I-insulin binding to liver membranes. Liver membranes (100 μg/ml) were incubated with ^{125}I-insulin (0.2 ng/ml) in the presence of variously diluted pepsin-digested anti-insulin serum. Results were expressed as a percent of control (100) in the presence of pepsin-digested non-immunized guinea-pig serum. Each point is the mean of triplicate determinations

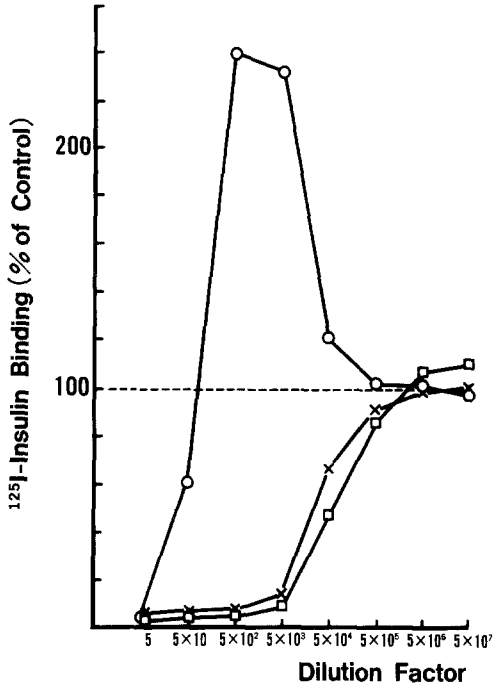

Fig. 6. Effects of non-immunized guinea pig serum and nonimmunized guinea pig IgG on AIAB enhanced ^{125}I-insulin binding to liver membranes. Liver membranes (100 μg/ml), ^{125}I-insulin (0.2 ng/ml) and variously diluted AIAB were incubated in the absence (○) or presence of non-immunized guinea pig serum (□, final dilution, 1:5) or non-immunized guinea pig IgG (x, final concentration, 200 μg/ml). As a control, liver membranes and ^{125}I-insulin were incubated with non-immunized guinea pig serum. Results were expressed as a percent of control (100). Each point is the mean of triplicate determinations

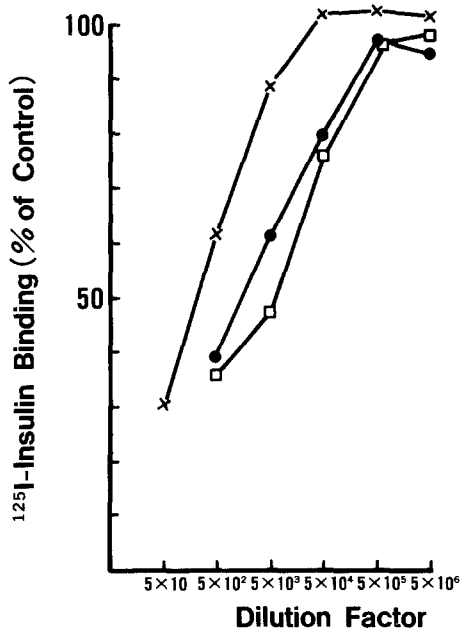

Fig. 7. Effects of non-immunized guinea pig IgG and anti-IgG sera on ^{125}I-insulin binding to liver membranes in the presence of AIAB (final dilution, 1:500). Liver membranes (100 μg/ml), ^{125}I-insulin (0.2 ng/ml) and AIAB (1:500) were incubated in the presence of variously diluted non-immunized IgG (x, original concentration, 20 mg/ml), Fc fragment-specific anti-IgG serum (●) or H&L-chain-specific anti-IgG serum (□). As a control, liver membranes and ^{125}I-insulin were incubated with AIAB alone (1:500). Results were expressed as a percent of control (100). Each point is the mean of triplicate determinations

To characterize these Mr = 62,000 and 27,000 bands, ^{125}I-insulin was cross-linked to AIAB in the absence of liver membranes. SDS-PAGE of these preparations revealed the two same bands (Fig. 4, D-F) as were revealed in the presence of liver membranes (Fig. 4, A-C). This result indicates that Mr = 62,000 and 27,000 bands correspond to the heavy and the light chain of anti-insulin IgG molecule respectively.

There is a quantitative difference of the 62K and the 27K bands in lanes A to C and lanes D to F (Fig. 4), because the amounts of radioactivity applied to the lanes D to F (without liver membranes) is much smaller than that of the lanes A to C (with liver membranes).

We then examined the possibility that insulin-AIAB complex might bind to the Fcγ receptor of the liver membrane [15]. Figure 5 shows the effect of pepsin-digested anti-insulin serum on ^{125}I-insulin binding to liver membranes. ^{125}I-insulin binding was inhibited by pepsin-digested serum in a dose dependent manner and ^{125}I-insulin binding was no longer enhanced.

We further examined the effects of non-immunized guinea pig serum, non-immunized guinea pig IgG, H&L-chain-specific anti-IgG serum, Fc fragment-specific anti-IgG serum and protein A on AIAB-induced enhancement of ^{125}I-insulin binding. When liver membranes, ^{125}I-insulin and AIAB were incubated in the presence of non-immunized guinea pig serum (final dilution, 1:5) or non-immunized guinea pig IgG (final concentration, 200 μg/ml), the enhancing effect of AIAB was completely abolished (Fig. 6). The formation of the ^{125}I-insulin-AIAB complex measured by dextran-coated charcoal method [16] was not significantly changed by the presence of non-immunized guinea pig serum or IgG (data not shown).

Figure 7 shows the effects of non-immunized IgG, Fc fragment-specific anti-IgG serum and H&L-chain-specific anti-IgG serum on the enhancing effect of AIAB. ^{125}I-insulin binding to liver membranes in the presence of AIAB (final dilution, 1:500) was inhibited by these materials in a dose-dependent manner. ^{125}I-insulin bound to AIAB (final dilution, 1:500) was about 80% of the total ^{125}I-insulin added and was not affected by IgG or anti-IgG sera (data not shown).

In the presence of protein A(20 μg/ml), H&L-chain-specific anti-IgG serum (final dilution, 1:500) or Fc fragment-specific anti-IgG serum (final diluaiton 1:500), enhancement of ^{125}I-insulin binding by AIAB was abolished (Fig. 8 a), and after cross-linking, SDS-PAGE and autoradiography showed that the labelling of Mr = 62,000 and 27,000 bands were also inhibited by these materials (Fig. 8 b).

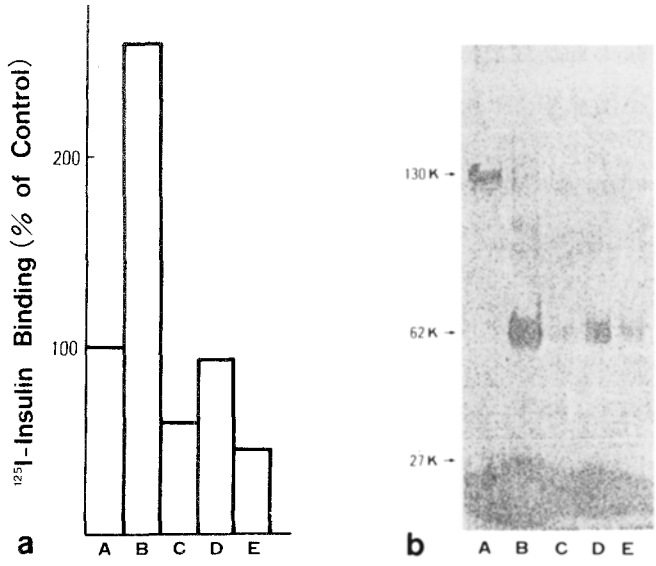

Fig. 8.a Effects of protein A, anti-Fc serum and H&L-chain-specific anti-IgG serum on AIAB-induced enhancement of insulin binding. ^{125}I-insulin (0.2 ng/ml), liver membranes (100 μg/ml) and AIAB (1:500) were incubated for 24 h at 4 °C in the absence (B) or presence of protein A (20 μg/ml, C), anti-Fc serum (1:500, D) and H&L-chain-specific anti-IgG serum (1:500, E). Insulin binding was determined after centrifugation and expressed as a percent of control (100) in the absence of AIAB and any of those three materials described above (A). Each column shows the mean of duplicate determinations. **b** Electrophoretic analysis of the effects of protein A, anti-Fc serum and H&L-chain-specific anti-IgG serum on AIAB-induced enhancement of insulin binding. ^{125}I-insulin (10 ng/ml), liver membrane (400 μg/ml) and AIAB (1:500) were incubated for 24 h at 4 °C in the absence (B) or presence of protein A (20 μg/ml, C), anti-Fc serum (1:500, D) and H&L-chain-specific anti-IgG serum (1:500, E). As a control study, ^{125}I-insulin and liver membrane were incubated without any other materials described above (A). Cross-linking, SDS PAGE and autoradiography were performed after incubation. Result corresponds to one representative experiment

Discussion

^{125}I-insulin binding to liver membranes was enhanced by AIAB at optimal concentrations when it was estimated by the radioactivity in the membrane pellets. This agrees with the results of Shechter et al. [2]. However, using the methods of covalent cross-linking of ^{125}I-insulin to the insulin receptor and AIAB, we clearly showed that ^{125}I-insulin binding to the α-subunit of the liver membrane insulin receptor was only inhibited by AIAB in a dose dependent manner. More importantly, SDS-PAGE, under reducing conditions and autoradiography, revealed Mr = 62,000 and 27,000 bands at the concentrations of AIAB which markedly increased ^{125}I-insulin binding to liver membranes. These two bands were found to correspond to the heavy and the light chain of anti-insulin IgG molecule respectively.

It is not likely that the association of insulin radioactivity to the light and heavy chain of the AIAB is an artifact of the cross-linking reaction because these two bands were not observed in IM-9 cells and RBC ghosts under the same experimental condition.

These results suggest that insulin-AIAB complex might bind to the liver membrane through the Fcγ receptor (15). The following findings, although they provide indirect evidence, are in support of this hypothesis. First, pepsin digested AIAB, which seemed to be separated to $(Fab')_2$ and Fc fragments of the IgG molecules [9], no longer enhanced the insulin binding. This result indicates that the Fc portion of anti-insulin IgG might play some role on the enhancement of insulin binding to liver membranes. Second, in IM-9 cells and HRBC ghosts, which have no Fcγ receptor [17, 18], AIAB-induced enhancement of the insulin binding was not observed and SDS-PAGE did not reveal ^{125}I-labelled heavy and light chains of IgG which were observed in liver membranes. Third, protein A, which binds to the Fc portion of the IgG molecule, inhibited the AIAB-induced enhancement of insulin binding and also inhibited the appearance of the heavy and the light chains in the autoradiography. Finally, non-immunized guinea pig serum or non-immunized guinea pig IgG completely abolished the enhancing effect of AIAB. This indicates that ^{125}I-insulin-AIAB complex could no longer bind to the Fcγ receptor because the Fcγ receptors were occupied and saturated by excess non-immune IgG.

This possibility is supported by the finding that ^{125}I-insulin binding in the presence of AIAB(1:500) was inhibited by non-immune IgG in a dose-dependent manner. Since ^{125}I-insulin binding to AIAB was not affected by non-immune IgG or anti-IgG sera, it is not likely that the inhibition of the enhancing effect of AIAB is due to the inhibition of the formation of ^{125}I-insulin-AIAB complex. Further, we confirmed that the inhibition of the enhancing effect of AIAB by protein A and anti-IgG sera was well correlated to the decreased density of the 62K and 27K bands.

It seems that these anti-IgG sera competed for Fcγ receptors with ^{125}I-insulin-AIAB complex as non-immune IgG. However, the reason is unclear why ^{125}I-insulin binding in the presence of AIAB (1:500) was inhibited by these anti-IgG sera. If ^{125}I-insulin-AIAB-anti-IgG antibody complex may be formed, it may bind to the Fcγ receptor through the Fc portion of anti-IgG antibody. It is possible that the spacial configuration of the Fc portion of anti-IgG by combination to insulin-AIAB complex interfered with the binding of Fc portion to the Fcγ receptor site as suggested by Khokher et al. [19]. SDS-PAGE and radioautography revealed no radioactive bands at an AIAB dilution of 1:5 (Fig. 3, B). In this condition, there was an excess of AIAB, and it is likely that Fcγ receptors were saturated by free AIAB which had not bound to ^{125}I-insulin.

We could not find the radioactive bands which might represent the ^{125}I-insulin-AIAB-Fcγ receptor complex in SDS-PAGE and autoradiography. There is no report up to date about the identification of the Fcγ receptor by using the cross-linking method. It is possible that the cross-linking reagent could not get access to the reaction sites of both AIAB and the Fcγ receptor to

which it might be bound. The reason for this, however, is not known, and further investigation is necessary.

In conclusion, our studies demonstrate that AIAB-induced enhancement of insulin binding to liver membranes is not due to the increased binding to the insulin receptor, but probably due to the binding of the insulin-AIAB complex to the Fcγ receptor of the liver membrane. In the presence of AIAB, insulin binding studies estimated by the radioactivity in the membrane pellets do not accurately reflect the insulin binding to the insulin receptor itself. However, it is still possible that very low concentrations of AIAB, at which insulin binding to the insulin receptor is not nearly inhibited, enhance the biological activity of coexisting insulin in intact cells as observed by Kahn et al. [1].

The evidence obtained for our hypothesis, however, is indirect because we could not demonstrate the cross-linking of the insulin-AIAB complex to the Fcγ receptor.

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