Proc. Natl. Acad. Sci. USA
Vol. 75, No. 5, pp 2443–2447, May 1978
Immunology

Use of anti-idiotypic antibodies as cell-surface receptor probes

(retinol-binding protein/insulin receptor/insulin effect)

KARIN SEGE AND PER A. PETERSON

Institute of Medical and Physiological Chemistry, Biomedical Center, University of Uppsala, Uppsala, Sweden

Communicated by Donald F. Steiner, March 10, 1978

ABSTRACT **Anti-idiotypic antibodies have been raised against antibodies to retinol-binding protein (RBP) and to insulin. After absorption the anti-idiotypic antibodies recognized the antigen-combining sites of the antibodies used as the immunogen but of no other antibodies. Some of the anti-idiotypic antibodies raised against antibodies to RBP bound specifically to rat intestine epithelial cells, which have a physiological cell-surface receptor for RBP. The RBP receptor mediates the uptake of retinol from RBP to the cells. This uptake was abolished in a concentration-dependent manner by the anti-idiotypic antibodies, which obviously competed with RBP for binding to the receptor.**

Anti-idiotypic antibodies against antibodies to insulin inhibited the binding of ^{125}I-labeled insulin to isolated rat epididymal fat cells, whereas anti-idiotypic antibodies raised against antibodies to RBP had no effect. Furthermore, on interacting with young rat thymocytes, anti-idiotypic antibodies against antibodies to insulin stimulated the uptake by the cells of α-aminoisobutyric acid, thereby mimicking the effect of insulin.

These results suggest that in some cases anti-idiotypic antibodies may be useful tools in elucidating structure–function relationships for cell-membrane receptors.

The immune system recognizes foreign substances by generation of antibodies that display an almost endless variety of combining sites. Thus, even a small, chemically well-defined molecule may, on immunization, give rise to stimulation of about 100 antibody-forming clones. Each clone produces a specific antibody with a unique amino acid sequence in the variable region (1). Due to the enormous variability in the antibody population, it seemed possible that among all the different antibodies that are elicited as a result of the immunization with, e.g., a hormone, a few antibodies might recognize that hormone in a fashion that mimics the way a physiological receptor recognizes the hormone. If so, the combining sites of such receptor-like antibodies might display structural features in common with the hormone-binding part of the receptor. Consequently, *a few* of a second set of antibodies raised against the combining sites, the idiotopes, of the "receptor-like" antibodies may, in their variable portions, be similar to those structures of the hormone that bind to the physiological receptor. Among such second sets of antibodies, anti-idiotypic antibodies, some would presumably interact with the hormone receptor in a hormone-like fashion.

To test the above reasoning we have raised anti-idiotypic antibodies against antibodies to retinol-binding protein (RBP) and to insulin. Since cell-surface receptors for RBP (2) and insulin (for a review, see ref. 3) exist, the possible binding of the anti-idiotypic antibodies to these receptors has been explored. This communication presents data that demonstrate that such anti-idiotypic antibodies may become useful tools in elucidating the structure and function of cell-surface receptors.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Antisera. The antiserum against β_2-microglobulin was the same as that used earlier (4). A rabbit antiserum was raised against highly purified, papain-solubilized rat transplantation (Ag-B) antigens.* Sprague–Dawley rats were immunized with bovine insulin by a described procedure (5). After several booster injections the rats were exsanguinated and the serum was collected from 10 animals. Specific antibodies against insulin were isolated by immunosorbent purification on a Sepharose-4B column to which insulin had been covalently attached (6). The pooled rat antibodies to insulin in Freund's complete adjuvant were injected into two rabbits which were boosted with the antigen in Freund's incomplete adjuvant three times on alternate weeks. Preimmune sera were obtained from the rabbits and were used as control sera throughout. The immunization schedule for obtaining anti-idiotypic antiserum against antibodies to RBP was identical to that described earlier (7).

IgG fractions from the anti-idiotypic antisera were isolated by affinity chromatography on Sepharose-4B columns containing covalently bound *Staphylococcus aureus* protein A (8). To remove antibodies directed against common determinants on rat IgG we subjected the anti-idiotypic IgG fractions to immunosorbent chromatography on Sepharose-4B columns containing covalently bound rat IgG. The rat IgG used in each case was isolated by protein A column affinity chromatography from the breakthrough fractions obtained after immunosorbent purification of the rat antisera (see above). In this way we ascertained that antibodies in the anti-idiotypic IgG fractions directed against subgroup-specific rat IgG antigenic determinants were removed. IgG fractions from the preimmune sera of the rabbits were processed in an identical fashion.

Prior to use the anti-idiotypic IgG fractions were passed over affinity chromatography columns containing covalently bound insulin and RBP to eliminate possible antibodies that might react directly with the proteins. The IgG in the breakthrough fractions of the columns were finally subjected to gel chromatography on columns of Sephadex G-200 to eliminate possible traces of protein that may have leaked from the affinity chromatography columns. The IgG fractions did not contain measurable amounts of insulin or RBP, as determined by sensitive radioimmunoassay procedures (4).

Radioactive Labeling of Proteins. Bovine insulin (Sigma Chemical Co.) was labeled with ^{125}I as described (9). The regular chloramin-T procedure for iodine labeling was used in all other cases (10). [^{3}H]Retinol (New England Nuclear, Dreieichenhein, Germany) was introduced into RBP as reported elsewhere (11).

Measurements of IgG Binding and [^{3}H]Retinol Uptake by Intestine Epithelial Cells. Intestine epithelial cells were isolated from Sprague–Dawley rats essentially as described (11).

Abbreviations: RBP, retinol-binding protein; AIB, α-aminoisobutyric acid.

* S. Kvist, L. Rask, and P. A. Peterson, unpublished data.

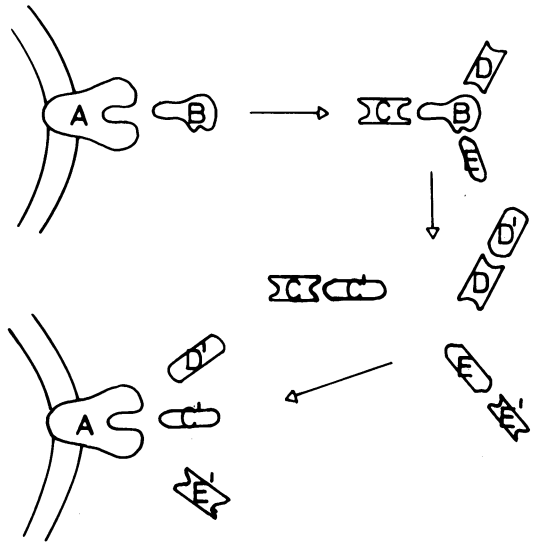

FIG. 1. Schematic representation of the rationale behind the experiments. See text for explanation.

Cells were incubated with the IgG fractions at 4° in 0.2 ml of buffer containing 20 mM Tris·HCl, pH 7.4/0.12 M NaCl/3 mM K_2HPO_4/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mg of bovine serum albumin per ml. After 45 min the cells were washed three times in the buffer and resuspended in the original volume; ^{125}I-labeled protein A from *Staphylococcus aureus* was then added (12). After 15 min of incubation, duplicate 100-μl aliquots were added to 250 μl of buffer in a micro test tube and centrifuged at 10,000 × *g* for 2 min in a Beckman 152 microfuge. The supernatants were aspirated and radioactivity was measured on the tips of the tubes, which contained the cell pellets.

[^{3}H]Retinol uptake from RBP to isolated intestine epithelial cells was carried out by the procedure outlined in detail elsewhere (11).

Insulin Binding to Fat Cells. Rat epididymal fat cells were isolated essentially by the procedure of Gliemann (13). ^{125}I-Labeled insulin was bound to the cells by following the protocol of Cuatrecasas (14).

α-Aminoisobutyric Acid (AIB) Uptake by Rat Thymocytes. Young rat thymocytes were isolated and the uptake of [^{14}C]AIB (Amersham, England) was measured as described by Goldfine *et al.* (15).

Other Methods. Concentration of proteins was estimated by measuring the absorbance at 280 nm related to the molar absorption coefficients of the proteins. Indirect immunoprecipitations with formalin-treated *Staphylococcus aureus* Cowan I were done as described elsewhere (16). Fab and Fc fragments were isolated as detailed (17). RBP was purified from human serum (18).

RESULTS

The rationale behind the experiments is schematically depicted in Fig. 1. The ligand *B* is specifically recognized by its membrane-bound receptor *A*. If antibodies are raised against *B*, several different clonotypes will be produced. However, only a few clonotypes (*C*) will interact with the receptor-fitting structure of ligand *B*, whereas the remainder of the antibodies (*D*, *E*, etc.) will bind to irrelevant parts of the ligand. If anti-idiotypic antibodies (*C'*, *D'*, *E'*, etc.) are raised against the antibodies to *B* (*C*, *D*, *E*, etc.), a few of the anti-idiotypic antibodies belonging to the *C'* subset may be similar enough in their combining sites to ligand *B* to bind to the receptor *A*. No such antibodies should be present in the *D'* and *E'* subsets. As a consequence, *C'* anti-idiotypic antibodies and ligand *B* will compete for the same site on receptor *A* and exhibit mutually exclusive binding.

Specificity of Anti-Idiotypic Antibodies. Fab and Fc fragments were isolated from the specific rat antibodies against insulin and RBP. Mixtures of the two types of Fab fragments, labeled with ^{125}I and ^{131}I, respectively, were separately incubated with each one of the anti-idiotypic IgG fractions and subjected to gel chromatography on columns (100 × 1 cm) of Sephadex G-200 equilibrated with 20 mM Tris·HCl, pH 8.0/0.15 M NaCl. When the mixtures contained anti-idiotypic antibodies raised against antibodies to insulin, no Fab fragments against RBP bound to the anti-idiotypic antibodies since all of the ^{125}I radioactivity was found at the elution position typical for free Fab fragments. In contrast, most of the Fab fragments against insulin bound to the anti-idiotypic antibodies since more than 80% of the ^{131}I radioactivity emerged close to the void fraction. The radioactive Fab fragments against RBP and insulin displayed the reciprocal elution behavior when the anti-idiotypic IgG fraction was directed against the antibodies to RBP. Control experiments showed that neither of the anti-idiotypic IgG fractions reacted with ^{125}I-labeled Fc fragments. IgG from the preimmune rabbit sera did not react with the labeled Fab fragments directed against insulin and RBP. These results indicate that the anti-idiotypic IgG fractions recognized only antigenic determinants present on those rat antibodies that had been used for the immunizations.

To show that the anti-idiotypic antibodies were indeed directed against the idiotopes of the rat Fab fragments we subjected mixtures containing labeled Fab fragments, anti-idiotypic IgG fractions, and varying concentrations of RBP and insulin to indirect immunoprecipitation. RBP but not insulin abolished the interaction between the anti-RBP Fab fragments and the anti-idiotypic antibodies in a concentration-dependent manner. The binding of the Fab fragments against insulin to the anti-idiotypic IgG fractions was completely inhibited by insulin, whereas RBP had no effect on the interaction. Thus, it seems reasonable to conclude that the anti-idiotypic antibodies displayed reactivity only against the antigen-binding sites of the Fab fragments.

Anti-Idiotypic Antibodies against Antibodies to RBP Bind to the Surface of Intestine Epithelial Cells. Intestine epithelial cells have cell-surface receptors for RBP (2, 11). Tests were therefore done to examine if any anti-idiotypic antibodies against antibodies to RBP might be similar enough to the RBP to bind to the receptor. To this end the IgG fraction of the anti-idiotypic antiserum and IgG isolated from the preimmune serum were separately incubated with rat intestine epithelial cells and the amount of IgG bound to the cells was measured. Fig. 2 shows that IgG from the anti-idiotypic antiserum bound significantly better to the intestine epithelial cells than did the control IgG from the preimmune serum. The anti-idiotypic antibodies probably displayed a rather low binding strength since relatively large quantities of IgG had to be used to saturate the available sites on the cell surface.

If the significantly higher binding of IgG from the anti-idiotypic antiserum compared to the control IgG was due to anti-idiotypic antibodies recognizing the RBP receptor, this binding should be abolished by antibodies against RBP (i.e., *C* in Fig. 1 should impede the binding of *C'* to *A*). Fig. 3 shows that this was indeed the case. The antibodies against RBP did not, however, significantly affect the background binding of the IgG from the preimmune serum. That the antibodies to RBP blocked the binding of IgG from the anti-idiotypic antiserum

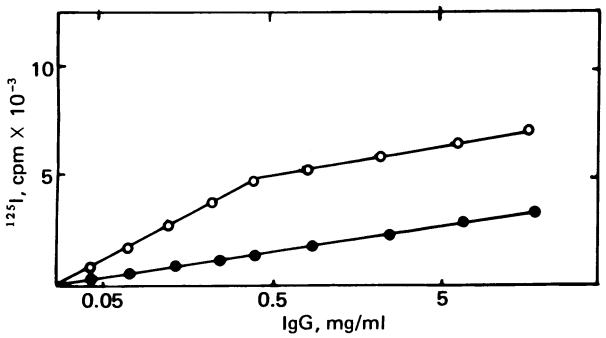

FIG. 2. Binding of anti-idiotypic antibodies against antibodies to RBP to rat intestine epithelial cells. IgG fractions from the anti-idiotypic antiserum (○) and the preimmune serum (●) were incubated with 5×10^5 cells. After washing, the IgG remaining bound to the cells was estimated by addition of excess ^{125}I-labeled protein A from *Staphylococcus aureus*. The values are the means of duplicate analyses.

in a specific manner is evident since antibodies against an irrelevant antigen (β_2-microglobulin) did not have any effect (Fig. 3). From this set of experiments it seems reasonable to conclude that some anti-idiotypic antibodies raised against antibodies to RBP bind to the surface of intestine epithelial cells.

Anti-Idiotypic Antibodies against Antibodies to RBP Impede RBP-Mediated [^{3}H]Retinol Uptake of Intestine Epithelial Cells. RBP interacts only in a transitory fashion with its receptor on the cell surface (2, 11). Available evidence suggests that on binding to the receptor RBP simultaneously gives up retinol and becomes structurally modified so that it cannot interact any longer with the receptor (11). To examine if the anti-idiotypic antibodies and RBP compete for binding to the same cell-surface structure (i.e., if *B* and *C'* both bind to *A* of Fig. 1) we investigated the influence of the anti-idiotypic antibodies on the RBP-mediated uptake of retinol by the intestine epithelial cells. Fig. 4 shows that the anti-idiotypic IgG fraction impeded the RBP-mediated uptake of [^{3}H]retinol by the cells. When the anti-idiotypic IgG fraction was passed over

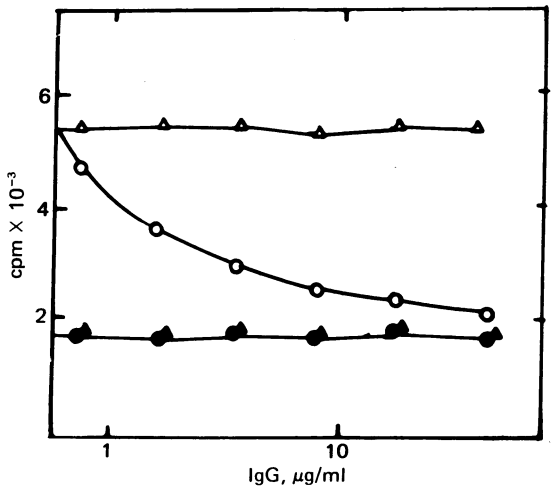

FIG. 3. Inhibition of the binding of anti-idiotypic antibodies to rat intestine epithelial cells by antibodies to RBP. The IgG fractions from the anti-idiotypic antiserum (open symbols) and the preimmune serum (solid symbols) were used at a concencentration of about 0.4 mg/ml. These IgG fractions were preincubated with various amounts of specific rat antibodies directed against RBP (○,●) or β_2-microglobulin (△,▲). Otherwise the experiment was performed as described in the legend of Fig. 2.

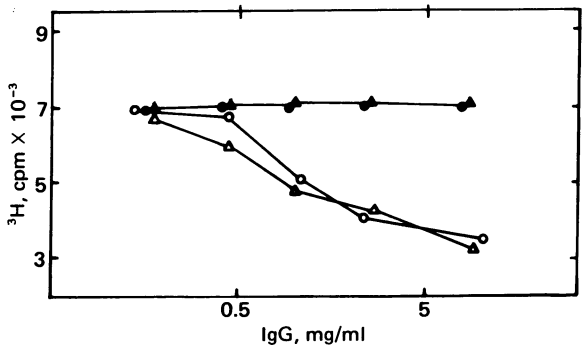

FIG. 4. Inhibition of the RBP-mediated uptake of [^{3}H]retinol by anti-idiotypic antibodies against antibodies to RBP. The incubation mixtures contained 5×10^5 cells, 4 μg of [^{3}H]retinol-containing RBP, and various concentrations of IgG in a total volume of 0.2 ml. ○, Anti-idiotypic IgG fraction; ●, anti-idiotypic IgG fraction that emerged in the breakthrough fraction from a column containing antibodies to RBP; △, anti-idiotypic IgG fraction obtained in the breakthrough fraction after passage of the material over an immunosorbent column containing antibodies to β_2-microglobulin; ▲, IgG fraction from a rabbit antiserum against β_2-microglobulin.

an immunosorbent column containing antibodies against RBP, the IgG in the breakthrough fraction did not exhibit the blocking effect (Fig. 4), suggesting that it was indeed the anti-idiotypic antibodies against the antibodies to RBP that competed with RBP for binding to the receptor. This interpretation was further reinforced by the observation that when the antiidiotypic IgG fraction was passed over an immunosorbent column containing antibodies against β_2-microglobulin, all of the inhibiting activity was recovered in the breakthrough fraction. Likewise, any type of antibodies will not impede the RBP-mediated uptake of [^{3}H]retinol, as shown by the fact that antibodies against β_2-microglobulin had no measurable effect on uptake of [^{3}H]retinol by the cells. Thus, these data are consistent with the view that an anti-idiotypic antiserum raised against antibodies to RBP may contain some antibodies that recognize the RBP raceptor on the surface of intestine epithelial cells.

Anti-Idiotypic Antibodies against Antibodies to Insulin Bind to a Cell-Membrane Insulin Receptor. To examine if the anti-idiotypic antiserum raised against highly purified antibodies against insulin contained antibodies that recognized the cell-membrane insulin receptor we performed competition experiments with insulin and the anti-idiotypic IgG fractions. Thus, the binding of ^{125}I-labeled insulin to fat cells was measured in the presence of IgG isolated from the anti-idiotypic antiserum. Fig. 5 shows that the IgG fraction inhibited the binding of the labeled insulin. No such inhibition was noted when IgG from the anti-idiotypic antiserum raised against antibodies to RBP was used. The figure also shows that the inhibiting activity in the IgG fraction from the anti-idiotypic antiserum raised against antibodies to insulin could be eliminated by passing the IgG fraction over an immunosorbent column containing antibodies to insulin, but no such effect was obtained with a column containing antibodies to RBP. This observation makes it reasonable to conclude that the anti-idiotypic antiserum raised against antibodies to insulin contained some antibodies that crossreacted with the insulin receptor on the rat epididymal fat cell membrane.

Anti-Idiotypic Antibodies against Antibodies to Insulin Mimic Insulin in Their Effect on Thymocyte AIB Uptake. From the reasoning detailed in Fig. 1 it is evident that anti-idiotypic antibodies, if able to bind to the cell-surface receptor

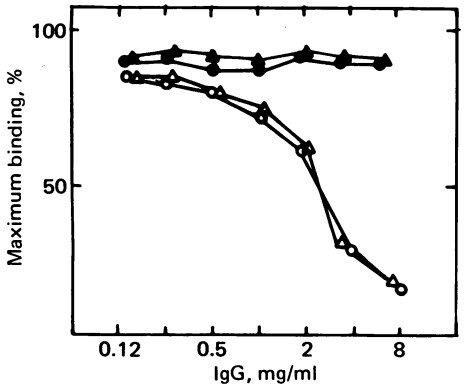

FIG. 5. Inhibition of the binding of ^{125}I-labeled insulin to epididymal fat cells by anti-idiotypic antibodies against antibodies to insulin. The incubation mixtures contained 6×10^4 rat epididymal fat cells, ^{125}I-labeled insulin to ensure 90% of the maximal specific binding (tested in a separate experiment), and various concentrations of IgG. The anti-idiotypic IgG fraction against antibodies to insulin (○) was passed over immunosorbent columns containing antibodies to insulin (●) and to RBP (△), respectively. The breakthrough fractions from the columns were used in the experiment. An IgG fraction from the anti-idiotypic antiserum against antibodies to RBP served as the control (▲).

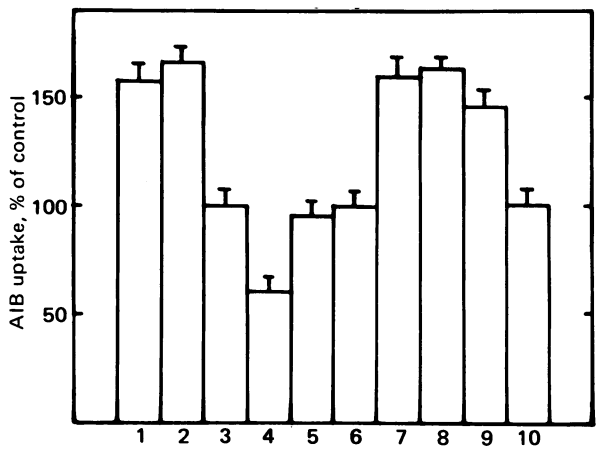

FIG. 6. Effect of anti-idiotypic antibodies against antibodies to insulin on AIB uptake by young rat thymocytes. The cells (6×10^7/ml) were incubated for 2 hr with 5 μg of insulin per ml or with the IgG fractions at a concentration of 5 mg/ml. Dose-response experiments showed that IgG at the concentration used gave maximal AIB uptake. The uptake of AIB in the absence of insulin served as the control. (*1*) Anti-idiotypic IgG fraction from rabbit no. 1; (*2*) anti-idiotypic IgG fraction from rabbit no. 2; (*3*) normal rabbit IgG fraction; (*4*) IgG fraction against AgB antigen; (*5*) anti-idiotypic IgG fraction from rabbit no. 1 passed over a column containing antibodies to insulin; (*6*) anti-idiotypic IgG fraction from rabbit no. 2 passed over a column containing antibody to insulin; (*7*) anti-idiotypic IgG fraction from rabbit no. 1 passed over a column containing antibodies to β_2-microglobulin; (*8*) anti-idiotypic IgG fraction from rabbit no. 2 passed over a column containing antibodies to β_2-microglobulin; (*9*) insulin; and (*10*) control. The values are the mean ± SD of quadruplicate analyses.

A, should interact with the receptor in a ligand-like fashion. Thus, anti-idiotypic antibodies binding to the insulin receptor should interact with the insulin-binding domain of the receptor. If so, it is conceivable that the anti-idiotypic antibodies may exert an insulin-like effect on the cells. To test this possibility we examined AIB uptake by young rat thymocytes, which is stimulated by insulin (15), in the presence of the anti-idiotypic antibodies. Fig. 6 shows that IgG fractions of anti-idiotypic antisera from two separate rabbits increased the AIB uptake to the same extent as insulin, although the amounts of protein required for the stimulation differed by several magnitudes. Normal IgG or IgG from an antiserum directed against the AgB antigens had no effect or depressed the AIB uptake by the thymocytes. The stimulating activity in the IgG fractions from the anti-idiotypic antisera was removed by immunosorbent chromatography of the IgG fractions on a Sepharose 4B column containing covalently bound antibodies to insulin. The IgG fractions from the anti-idiotypic antisera retained, however, full stimulating activity in the AIB uptake assay after they had been passed over an immunosorbent column containing antibodies against β_2-microglobulin, demonstrating that it was the antiidiotypic antibodies in the IgG fractions that exhibited the stimulation of the AIB uptake. These data are, thus, consistent with the view that anti-idiotypic antibodies raised against antibodies to insulin, at least in this assay system, may exhibit an insulin-like effect.

DISCUSSION

The anti-idiotypic IgG fractions used in the present study seem to encompass at least some antibodies that display binding characteristics similar to the original antigen used for the immunizations. Since the interactions between the anti-idiotypic antibodies and the cell-surface receptors are abolished by antibodies raised against the protein antigens, it seems reasonable to conclude that it is the combining sites of the anti-idiotypic antibodies that bind to the receptors. Due to the procedure used to obtain the antibodies to the receptors, their interaction with the receptor should mimic that of the hormone. This expectation seems to be fulfilled by the observation that at least some of the anti-idiotypic antibodies against antibodies to insulin stimulate the uptake of AIB by young rat thymocytes. Other antibodies directed against protein unrelated to insulin did not display such an effect.

The observation that the anti-idiotypic antisera reacted only at high concentrations with the examined cell-surface receptors makes it difficult to analyze the interactions in quantitative terms. The data could, thus, be explained by the assumption that only very few antibodies met the structural requirements for the interaction or that a sizable number of the anti-idiotypic antibodies reacted, but poorly, with the receptors.

In considering the specificity of the anti-idiotypic antibodies it may appear astonishing that antibodies are produced against self-structures (i.e., cell-surface receptors). However, it has been well documented in other experimental systems that tolerance may be abrogated by the immunization with modified self-structures (see ref. 19). The "receptor-like" primary antibodies may, thus, be regarded as modified antigens. Once anti-idiotypic antibody-forming clones are activated, the cell-surface receptors may serve as the antigen so that an auto-immune reaction is induced. The present data do not provide any insight into this possibility, but it is tempting to suggest that this may be one of the mechanisms explaining the occurrence of antibodies to the insulin receptor in some diabetic patients (20). Moreover, antibodies that are directed against a cell-surface receptor exerting a hormone-like action are frequently encountered in thyreotoxicosis. The long-acting thyroid stimulator may, thus, in some cases perhaps arise as an anti-idiotypic antibody. In the immune system *per se* it has been shown that autogeneously produced antibodies to receptors can be functional in regulating immunity of the individual producing the antibodies to the receptor (21).

The present data suggest that the use of anti-idiotypic antibodies to explore the structure and function of cell-surface re-

ceptors may have some promise. However, it has to be established whether other protein and hormone antigens could be used to obtain anti-idiotypic antibodies of the desired specificity. It is also much too early to know whether all animals and all immunization protocols will be of use, and ways have to be devised to improve the titer of the anti-idiotypic antisera to make them useful reagents in procedures like indirect immunoprecipitations.

This work was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Society, and Nordisk Insulinfond.

1. Nisonoff, A., Hopper, J. E. & Spring, S. B. (1975) *The Antibody Molecule* (Academic, New York).
2. Peterson, P. A., Nilsson, S. F., Östberg, L., Rask, L. & Vahlquist, A. (1974) *Vitam. Horm.* (N.Y.) **32**, 181–214.
3. Kahn, C. R. (1976) *J. Cell Biol.* **70**, 261–286.
4. Peterson, P. A., Rask, L. & Lindblom, J. B. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 35–39.
5. Peterson, P. A., Evrin, P. E. & Berggård, I. (1969) *J. Clin. Invest.* **48**, 1189–1198.
6. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065.
7. Sege, K. & Peterson, P. A. (1978) *Nature* **271**, 167–168.
8. Hjelm, H., Hjelm, K. & Sjöquist, J. (1972) *FEBS Lett.* **28**, 73–76.
9. Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1833–1837.
10. Greenwood, F. C., Hunter, W. H. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123.
11. Rask, L. & Peterson, P. A. (1976) *J. Biol. Chem.* **251**, 6360–6366.
12. Dorval, G., Welsh, K. I. & Wigzell, H. (1974) *Scand. J. Immunol.* **3**, 405–411.
13. Gliemann, J. (1967) *Diabetologia* **3**, 382–388.
14. Cuatrecasas, P. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1264–1268.
15. Goldfine, I., Gardner, J. D. & Neville, D. M., Jr. (1972) *J. Biol. Chem.* **247**, 6919–6926.
16. Östberg, L., Sege, K., Rask, L. & Peterson, P. A. (1976) *Folia Biol. (Prague)* **22**, 372–373.
17. Vahlquist, A. & Peterson, P. A. (1973) *J. Biol. Chem.* **248**, 4040–4046.
18. Peterson, P. A. (1971) *J. Biol. Chem.* **246**, 34–43.
19. Ruoslahti, E. & Wigzell, H. (1975) *Nature* **255**, 5511, 716–717.
20. Flier, J. S., Kahn, C. R., Roth, J. & Bar, R. S. (1975) *Science* **190**, 63–65.
21. Rowley, D. A., Köhler, H., Schreiber, H., Kaye, S. T. & Lorbach, I. (1976) *J. Exp. Med.* **144**, 946–959.