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Autoantibodies to Insulin Receptor Spontaneously Develop as Anti-Idiotypes in Mice Immunized with Insulin

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Abstract. *Mice immunized with insulin developed antibodies to both insulin and the insulin receptor. The antibodies to insulin receptor displaced labeled insulin from insulin receptors and mimicked the actions of insulin in stimulating the oxidation of glucose and its incorporation into lipids, and in inhibiting lipolysis. The antibodies to insulin receptor could be blocked by or bound to the antibodies to insulin, and therefore were identified as anti-idiotypes. Thus, immunization against a hormone may activate spontaneously an idiotype–anti-idiotype network resulting in antibodies to the hormone receptor.*

Several diseases of man can be related to the action of autoantibodies binding to receptors on the surface of normal cells of the individual. For example, patients with Graves' disease have antibodies that abnormally stimulate the thyroid gland by activating receptors for thyroid-stimulating hormone (TSH) (*1*). These antibodies to TSH receptor cause hypersecretion of thyroid hormones and hyperthyroidism. Myasthenia gravis is caused by antibodies that bind to and block the receptor for acetylcholine at the neuromuscular junction (*2*). Another instance of autoimmunity to receptor is exemplified by those patients with antibodies to the insulin receptor who suffer from severe insulin-resistant diabetes (*3*). The pathophysiology of autoimmunity to hormone receptors is not well understood. How might an individual's immune system be triggered to produce antibodies that bind to seemingly normal membrane components?

One explanation for receptor autoimmunity is that it arises in the course of anti-idiotypic regulation of the immune response. Animals may be immunized against the variable regions of specific antibody molecules (idiotypes) and so produce anti-idiotypes, antibodies directed against idiotypes (*4*). Some anti-idiotypes recognize the combining site of the idiotype (*5*). Thus, either antigen or anti-idiotypes may bind to the combining sites of idiotypic antibodies. Jerne (*6*) proposed that the immune system may be regulated by a network in which antigen induces production of idiotypes which in turn induce anti-idiotypes that can feedback to shut off or modify the original idiotypic response. Antibodies to idiotypes can readily be prepared by immunizing animals against purified idiotypes; anti-idiotypes so produced have been shown to influence immune reactions (*7*). However, the spontaneous generation of anti-idiotypes in response to immunization against an antigen has seldom been detected (*8*), and therefore it has been difficult to demonstrate a physiological function for the hypothetical idiotype–anti-idiotype network.

Sege and Peterson (*9*) proposed that a hormone could be used as an antigen to stimulate idiotypic antibodies that recognize the hormone. Such antibodies might be used to induce anti-idiotypes that sterically fit the combining sites of the idiotypes. Thus, the anti-idiotypes could have a three-dimensional configuration similar to that of the hormone antigen, and such anti-idiotypes might be able to bind to the hormone receptor. Accordingly, Sege and Peterson immunized rabbits against rat antibodies to insulin and obtained anti-idiotypic antibodies that bound to rat insulin receptor.

We explored the possibility that animals immunized against insulin might spontaneously develop antibodies to their own insulin antibodies, and that some of these anti-idiotypes might interact with the insulin receptor. Our approach was to immunize mice against bovine or porcine insulins and to assay samples of their serum for the appearance of two factors: antibodies to insulin

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and antibodies to the insulin receptor that mimic the action of insulin. Antibodies to insulin were detected in mice that were genetically able to respond to the particular immunizing insulin (*10*), and these antibodies increased to a relatively high titer after the mice were given a booster injection of insulin. However, in about 70 percent of the mice we also detected a remarkably high level of insulin-like activity by observing the effects of the serum on isolated rat fat cells in vitro. Figure 1A shows that lipogenesis, the incorporation of labeled glucose into lipid, was linearly related to the amount of test serum added to a suspension of fat cells and that 50 percent of maximum stimulation of lipogenesis was obtained at 1.1 μl of test serum. Because insulin produced 50 percent of maximum stimulation at a concentration of 0.3 ng/ml in this assay (not shown), it can be calculated that the test serum contained insulinlike activity equivalent to 270 ng of insulin per milliliter. The test serum competed with insulin for insulin receptors and displaced labeled insulin from the fat cells (Fig. 1B). Control serum obtained from mice not immunized against insulin showed neither of these effects (Fig. 1, A and B). Mice genetically incapable of mounting an immune response to porcine or bovine insulin did not develop insulinlike activity in response to injection of these insulins; only the serum of mice that made antibodies to insulin developed insulinlike activity.

We found that the insulinlike activity was a property of immunoglobulin G (IgG) molecules. Serum samples were passed through chromatography columns of protein A bound to Sepharose. Protein A binds specifically to the Fc portion of antibodies, primarily of the IgG class (*11*). All of the insulinlike activity reflected by displacement of insulin (Fig. 1B), glucose oxidation (*12*), lipogenesis (Table 1 and Fig. 1A), and inhibition of lipolysis bound to and could be eluted from the protein A–Sepharose column (Table 1). The eluted material was identified as IgG by means of polyacrylamide gel electrophoresis (*13*). No insulinlike activity was detectable in the effluent that was not bound.

The IgG with insulinlike activity logically could be attributed to two different kinds of antibodies. Antibodies to insulin (idiotypes) complexed with insulin could be envisioned as binding to the insulin receptor because of the insulin component of the complex. In this case the antibody would recognize only the hormone, whereas the insulin would interact with its receptor on the fat cell. Alternatively, antibodies to idiotypes with the three-dimensional configuration of insulin might bind to the insulin receptor directly and thus function as antibodies to the receptor without the mediation of insulin. In experiments designed to discriminate between these possibilities we found that insulin and the antibodies with insulinlike activity seemed to interact with different sites on the insulin receptor. Mild trypsinization of fat cells, a treatment that greatly decreases the ability of the cells to respond to insulin (*14*), did not affect the ability of the fat cells to respond to the insulinlike antibody (Fig. 1C). The insulinlike effects of the lectin concanavalin A are also unaffected by treating target fat cells with low concentrations of trypsin (*15*). Hence, it is conceivable that the insulinlike antibodies interact with the same trypsin-resistant site on the receptor as does concanavalin A. In any case, these results argue against the possibility that insulin itself complexed to antibodies to insulin could have mediated the interaction between the insulin receptor and the antibodies with insulinlike activity. We directly analyzed this point by dissociating any immune complexes that might exist between insulin and antibodies to insulin and separating the dissociated insulin by gel chromatography (Table 1). We found that 85 percent of the insulinlike lipogenic activity was associated with the high molecular weight material that was free of insulin. Hence, there was no evidence that the insulinlike activity was related to immune complexes.

Evidence that the insulinlike antibodies to insulin receptor were anti-idiotypes was derived from the finding that lipogenesis was inhibited by adding affinity-purified mouse antibodies to insulin to the serum (Table 1). We also separated physically the insulinlike antibodies from the insulin antibodies. When we passed the two types of antibodies through a column of agarose covalently bound to either normal guinea pig serum or guinea pig antiserum to insulin, the mouse insulin antibodies were not re-

Table 1. Insulin antibody and insulinlike lipogenic activities of serum from test mice immunized against insulin. The titer of the insulin antibodies was determined as described (*27*). Adipocytes were prepared and lipogenesis was measured as described in the legend to Fig. 1.

Treatment of test serum	Serum fraction	Insulin antibody titer (dilution^{-1})	Lipogenesis (microliters per 50 percent activity)	Activity of serum fraction relative to activity of whole serum (%)	
				Insulin antibody	Lipogenesis
None	Whole	10^4	1.1	100	100
Dissociation of immune complexes*	Void volume	N.D.	1.3	N.D.	85
Addition of mouse antibody to insulin (12 μg/ml)†	Whole	N.D.	2.2	N.D.	50
Protein A column‡	Effluent	N.D.	>10	N.D.	0
	Eluate	N.D.	1.1	N.D.	100
Insulin antiserum column‡	Effluent	10^4	1.6	100	70
	Eluate	<1.0	3.7	0	30
Insulin column‡	Effluent	<1.0	1.3	0	85
	Eluate	10^4	>10	100	0

*Test serum (50 μl) was acidified to *p*H 2.5. Some [^{125}I]insulin (40,000 count/min) was loaded on a Sephadex G-100 column (20 by 0.5 cm) which was preequilibrated and developed with 0.1*M* acetic acid and 0.2 percent bovine serum albumin (BSA) (*p*H 2.5). The void volume fractions prior to these that contained radioactive insulin were pooled, neutralized, and examined for bioactivity. †Mouse IgG antibody to insulin was adsorbed on an agarose-insulin column, eluted with 0.1*M* acetic acid and neutralized with solid $NaHCO_3$. This fraction blocked insulin-mediated lipogenesis at a molar ratio of IgG to insulin of 2000 to 1 (*22*). ‡Test serum (30 μl) was loaded on a protein A–Sepharose column (Pharmacia; 1.0 by 0.5 cm) that was preequilibrated and washed with a buffer consisting of Krebs-Ringer bicarbonate and 2 percent BSA (*p*H 7.4). A fraction of 2.0 ml was collected (effluent). The column was then washed with 0.1*M* acetic acid, and a 2.5-ml fraction (eluate) was collected and immediately neutralized with solid $NaHCO_3$ to *p*H 7.4. Essentially the same procedure was applied to the insulin-agarose affinity column (insulin-succinyl-diaminodipropylamine–agarose), prepared as described (*23*) and to the insulin antiserum–agarose column [prepared from guinea pig antiserum to pork insulin (Miles-Yeda) covalently linked to Sepharose–polyacrylamide hydrazide (*24*)]. Control columns of Sepharose or agarose alone or nonrelevant serum covalently linked to agarose did not adsorb any of the activities identified in this table. Volumes of the fractions that emerged from the columns were measured, and the results computed as the percentage activity relative to that obtained from the test serum before column separation. The results are representative of experiments done with seven individual samples of test serum.

tarded at all, whereas 30 percent of the insulinlike activity was bound. This bound activity was quantitatively eluted from the column and the eluate contained essentially none of the antibody to insulin. Using another affinity column, made of insulin-agarose (Table 1), we found that only the antibodies to insulin bound to and could be eluted from this column.

These results indicate that anti-idiotypes can be generated spontaneously in response to immunization against antigen and lend support to the network hypothesis of Jerne (*6*). We suspect that our success in detecting spontaneous idiotypes can be attributed to the fact that we measured their insulinlike effects on the metabolism of fat cells. The immunological assays of anti-idiotypic antibodies now available probably lack the degree of amplification inherent in the metabolic assays that we used. It is not certain that anti-idiotypes function to regulate immune responses as proposed by Jerne (*6*), nor is it clear how idiotypes and anti-idiotypes can coexist and function in the same serum without neutralizing each other. However, this study does show that anti-idiotypes can participate in a chain of autoimmune reactions leading to autoimmunity to receptor. Murine, human, and ungulate insulins differ by very few amino acid substitutions (*16*) that are concentrated at the pole of the molecule, and this region apparently does not bind to the hormone receptor (*17*). Antibodies produced in response to one insulin cross-react fully with the other insulins (*10, 18*), including self-insulin. Hence, some if not most antibodies to insulin can recognize antigenic determinants on self-insulin and, thus, are autoantibodies. T lymphocytes can also recognize self-insulin and express autoimmunity to insulin (*19*). Spontaneous anti-idiotypes recognizing self-antibodies constitute a second element of autoimmunity. A third example of autoimmunity in the network is the interaction of these anti-idiotypes with the hormone receptor. A similar network of autoimmunity could account for spontaneous antibodies to hormone receptors in humans. Hence, some patients apparently suffering from autoimmunity to TSH or to insulin receptors may be expressing a primary autoimmune response against the hormones. Mice demonstrating very high levels of insulinlike activity appeared to be clinically healthy provided that they were allowed free access to food. Therefore, antibodies to insulin receptor may be clinically inapparent, in some humans as well as in mice. Humans treated with exogenous insulin often develop antibodies to insulin (*20*). Might not some of these persons also develop anti-idiotypes with antireceptor activity? How do men or mice adapt to such receptor antibodies so that these molecules remain clinically covert? What effects might such antibodies have on the clinical course of diabetes?

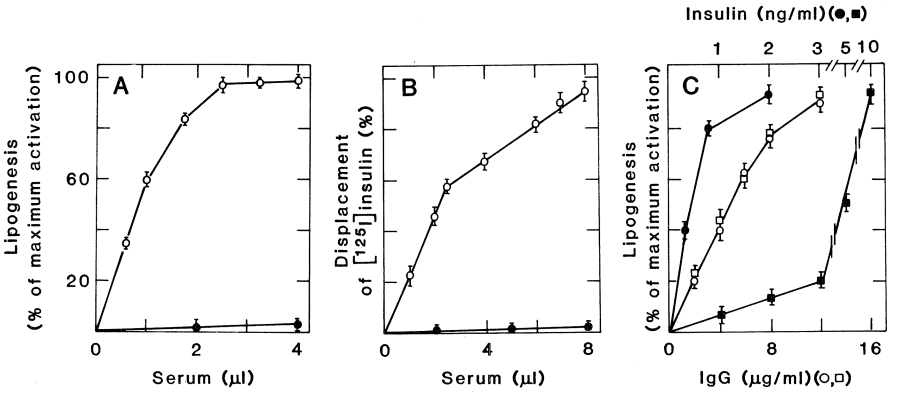

Fig. 1. Lipogenesis and displacement of labeled insulin from fat cells by mouse test serum and its IgG fraction. Mice (10 to 12 weeks of age) of strains BALB/c (H-2^d) or (BALB/c × C57BL/6)F_1 (H-2^d × H-2^b) were immunized with bovine or porcine insulin by injection in the hind foot pads with 25 μg of insulin emulsified in complete Freund's adjuvant (*10*). Three weeks later the mice received an intraperitoneal booster dose of the same insulin in adjuvant, and blood was obtained 10 days later. Control mice were immunized with complete Freund's adjuvant without insulin. Test (○) or control (●) serum samples were assayed for antibodies to insulin as described in Table 1 and for (A) lipogenesis or (B) displacement of [^{125}I]insulin. (A) From 120-g male Wistar rats, suspensions of adipocytes were prepared containing 0.5 × 10^4 cells per milliliter in Krebs-Ringer bicarbonate buffer (*p*H 7.4) containing 0.3 percent bovine serum albumin, as described (*25*). The adipocytes were incubated in plastic vials (5 ml per vial) for 2 hours at 37°C in 95 percent O_2 and 5 percent CO_2, with 0.2 m*M* [U-^{14}C]glucose and increasing concentrations of serum from test or control mice. The reaction was terminated by adding toluene-based scintillation fluid directly into the incubation vials and counting the extracted lipids (*26*). Maximum lipogenesis, obtained by incubation with 10 ng of insulin per milliliter, was 480 percent of control values without added hormone. Lipogenesis produced by added serum was computed as the percentage of activation relative to the maximum produced by 10 ng of insulin per milliliter. (B) Suspensions of adipocytes (3 × 10^5 cells per milliliter) were incubated with [^{125}I]insulin (0.3 ng/ml, 200,000 count/min-ng) and test or control serum samples in the above buffer for 1 hour at room temperature in a volume of 0.5 ml. The adipocytes were then separated from unbound hormone on Millipore (EGWP, 0.2 μm) filters, washed with ice-cold buffer, and counted for their radioactive content as described (*21*). The extent of binding was 1.7 fmole per 3 × 10^5 cells of which 76 percent was specific (displaced by 1 μ*M* native insulin). Results are expressed as percentage of displacement of specifically bound labeled insulin. Serum that had a relatively high titer of insulin bodies (10^{-3} dilution) but no insulinlike activity did not displace labeled insulin from fat cells (not shown). (C) Lipogenesis by IgG (○, □) and insulin (●, ■) in control (○, ●) and trypsinized (□, ■) fat cells. Immunoglobulin G from the test serum was isolated by adsorption on a Sepharose–protein A affinity column, as described in Table 1. The adsorbed IgG fraction was eluted with 0.1*M* acetic acid and neutralized with $NaHCO_3$. Trypsinization was carried out by exposing the fat cell suspension to trypsin (30 μg/ml) for 10 minutes at 37°C; ovoinhibitor (80 μg/ml) was then added (*14*). The results of (A), (B), and (C) are the means (± standard deviation) of triplicate samples of individual test and control serums. Similar results were obtained for 18 of 24 serum samples tested.

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12. The test serum and its IgG fraction (Table 1) also stimulated glucose oxidation and inhibited lipolysis. Glucose oxidation was carried out with suspensions of adipocytes (3 × 10^5 cells per milliliter) for 2 hours at 37°C with 0.2 m*M* [U-^{14}C]glucose. The amount of glucose converted to CO_2 was determined as described (*25*). Maximum glucose oxidation in the presence of insulin

was 3.5-fold higher than in the absence of the hormone. Assay of lipolysis was carried out for 3 hours in the absence of the hormone, and for 3 hours at 37°C in 5-ml vials containing 3×10^5 cells, 0.4 μM isoproterenol, and increasing concentrations of the test serum. Portions of the medium were then taken, bovine serum albumin was removed by trichloroacetic acid precipitation, and the glycerol content was determined by the triglyceride C-37 Rapid-Stat test (see *Pierce Catalog*, 1976). The amount of glycerol released was 12 and 140 nmole per 3×10^5 cells per 3 hours in the absence and the presence of 0.4 μM isoproterenol, respectively. Insulin at 10 ng/ml inhibited 87 percent of the glycerol released. The titer of antibodies to insulin was determined by a solid phase radioimmunoassay (*27*). Half-maximum activity was obtained at 1.0 μl and 1.5 μl of the test serum per milliliter for glucose oxidation and inhibition of lipolysis, respectively.

13. The fraction eluted from the protein A–Sepharose column was pure IgG as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and mercaptoethanol performed as described by U. K. Laemmli [*Nature (London)* **227**, 680 (1970)]. Only the heavy and light chains of IgG were detectable on the gel. No bands that corresponded to insulin were seen.
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