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Mouse Antibodies to the Insulin Receptor Developing Spontaneously As Anti-idiotypes

I. CHARACTERIZATION OF THE ANTIBODIES*

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Mice immunized to ungulate insulins were found to develop antibodies of two specificities: insulin antibodies that were mostly IgG1 and IgG2 antibodies that acted both as anti-idiotypes to specific mouse insulin antibodies and as antibodies to the insulin receptor. There was a negative association between the presence of anti-idiotypic receptor antibodies and insulin antibodies bearing the specific idiotype; the specific idiotypic antibodies were confined to the early phase of the primary response while the anti-idiotypic receptor antibodies were detected only after the idiotypic antibodies had disappeared. To map the insulin epitope that triggered the specific idiotypic response, we chemically altered the insulin molecule so as to inhibit its interaction with the insulin receptor. The altered insulins triggered high titers of antibodies binding to antigenic determinants on native insulin, but no anti-idiotypic receptor antibodies. Thus, the epitope responsible for the specific idiotypic-anti-idiotypic network was probably the part of the insulin molecule whose conformation is recognized by the insulin receptor.

In previous studies, we have observed that immunization of mice to ungulate insulin led to the development not only of antibodies to insulin, but of insulin-like antibodies that appeared to recognize and activate the insulin receptor (1, 2). As these receptor antibodies also bound to guinea pig anti-insulin antibodies, we concluded that they were probably anti-idiotypes to specific insulin antibodies. In other words, the receptor antibodies could have arisen in the mice as part of an idiotype-anti-idiotype network (3) in which the specific idiotypic antibody mimicked the structure of the insulin receptor (4). To prove this hypothesis, it was necessary to demonstrate the specific idiotype in the mice that developed the anti-idiotype and to show that the critical epitope on the insulin molecule was that portion of insulin that interacted with the insulin receptor. The present study was undertaken to establish these points. The results suggest that anti-idiotypic antibodies spontaneously developing as physiological components of an immune network (3) may function as receptor antibodies by virtue of their steric mimicry of the hormone antigen (4).

MATERIALS AND METHODS

Animals—Female mice of the (C3H/ebxC57BL/6)F1 hybrid strain were supplied by the Animal Breeding Center of this Institute or by Jackson Laboratories, Bar Harbor, ME, and immunized between 2 and 4 months of age. Male Wistar rats (70–100 g) used as a source of adipocytes originated from the colony of the Department of Hormone Research, Weizmann Institute.

Reagents—D[U-^{14}C]Glucose (4–7 mCi/mol) was purchased from New England Nuclear; collagenase Type I (134 units/mg) was from Millipore Corp., Worthington, NJ; and Sepharose-protein A affinity column was from Pharmacia, Uppsala, Sweden. Bovine insulin, acetic anhydride, succinic anhydride, and trinitrobenzenesulfonic acid were purchased from Sigma.

Preparation of Insulin Derivatives—Acetyl$_3$ insulin was prepared by cooling 10 ml of an insulin suspension (5 mg/ml) to 0 °C and adding 10-μl aliquots of acetic anhydride to the stirred solution over a period of 30 min. The solution was neutralized to pH 7.5 by the addition of 1 M NaOH, and the modified insulin went into solution. After adding 700 M excess of acetic anhydride, the protein solution was dialyzed in the cold for 24 h, first against 0.1 M hydroxylamine pH 7.5 (to deacetylate moieties other than amino groups), and then against three changes of water, and was then lyophilized. All three amino groups were modified, as judged by the absence of free amino groups in the trinitrobenzenesulfonic acid assay (5).

Succinyl$_3$ insulin was prepared by adding to the insulin suspension (5 mg/ml in H_2O) aliquots of solid succinic anhydride in excess. The reaction was carried out for 1 h at room temperature. The pH was kept at 7.5–8.0 by the addition of NaOH. The cleared protein solution was dialyzed for 24 h in the cold against three changes of water and lyophilized. Trinitrobenzene$_3$ insulin was prepared by adding 30 M excess of trinitrobenzenesulfonic acid to an insulin solution (1 mg/ml) in 0.1 M $NaHCO_3$. The reaction was maintained for 1 h at 37 °C. The protein solution was dialyzed against three changes of H_2O and lyophilized.

Immunization to Insulin—Mice were immunized by inoculation into each hind footpad of 25 μg of bovine insulin emulsified in complete Freund's adjuvant, as described (6). Control mice were immunized with the adjuvant alone. Sera were obtained for study at various times after primary immunization (primary serum). Some mice were boosted 3 weeks later with the same concentration of insulin in adjuvant or with adjuvant alone and bled at various times after boosting. Serum obtained 10 days after boosting is referred to as secondary serum. The results shown here were derived from the pooled sera of 15 mice. Study of sera from 10 individual mice produced essentially the same results. A solid phase radioimmunoassay was used to measure antibodies to insulin (7). The titer was computed as the reciprocal of the last $\log_{10}$ dilution that produced greater than twice the counts/min measured using control serum obtained from mice that had been immunized with adjuvant alone.

Affinity Purification of Insulin Antibodies—Mouse antibodies to insulin were affinity purified by applying 0.2 ml of serum to an insulin-agarose column (3 × 0.4 cm) prepared as described (8). The column was developed with 0.05 M sodium phosphate (pH 7.0). The first 3 ml were collected (effluent fraction), and the column was

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further washed with 10 ml of the same buffer. Elution was carried out using 0.01 M HCl, and 0.4-ml fractions were collected into tubes containing $NaHCO_3$ in amounts sufficient to neutralize the acidity. Fractions containing protein were pooled. Immunoglobulin concentration was determined by absorption using $\epsilon^{1\%}_{280} = 14$ (9). Protein A affinity purification of IgG2 antibodies was carried out using a Sepharose-protein A affinity column. IgG1 was washed from the column using 0.05 M sodium phosphate buffer (pH 7.0), and the bound IgG2 was eluted using 0.01 M HCl (10). An Ouchterlony assay of precipitation in agar (11) was used to identify the Ig class of purified insulin antibodies or of insulin-like antibody binding to protein A. Antisera to mouse IgG1, IgG2, IgG2A, IgG2B, IgA, and IgM were purchased from Meloy Lab. Inc., Springfield, VA.

Binding of ^{125}I-Insulin to Adipocytes—Rat adipocytes were prepared as described (12), and 1×10^5 cells were incubated in plastic culture tubes containing 0.35 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with 0.3% bovine serum albumin at 25 °C for 40 min together with ^{125}I-insulin (35,000 cpm/tube) and either native insulin or aliquots of sera or purified IgG. The cells were then separated from unbound hormone on Millipore filters (EGWP, 0.2 mμ), washed with ice-cold buffer, and counted for their radioactive content. Extent of binding was 1.7 fmol/3 × 10^5 cells of which 70% was specific (displaced by 1 μM native insulin).

Dissociation of Insulin-Antibody Complexes—Immune serum (0.2 ml) was incubated for 24 h at 4 °C with ^{125}I-insulin (250,000 cpm). The serum was acidified to pH 2.7 and applied to a Sephadex G-100 column (20 × 0.5 cm), equilibrated, and run with 0.1 M acetic acid containing 0.2% bovine serum albumin (pH 2.7). Fractions of 0.2 ml were collected into tubes containing $NaHCO_3$ in an amount sufficient to neutralize the pH. Aliquots of 20 μl were measured for their radioactive content or for their ability to stimulate lipogenesis. Lipogenesis is expressed in relative units in which 1 ml of immune serum was taken as 10,000 units.

Lipogenesis—The incorporation of [U-^{14}C]glucose into lipids was measured by incubating a 1% (v/v) fat cell suspension (0.5 ml in each small plastic vial) in Krebs-Ringer bicarbonate buffer (pH 7.4) with 0.7% bovine serum albumin and [U-^{14}C]glucose (final concentration 0.2 mM) at 37 °C, under CO_2/O_2 (5:95 v/v) atmosphere. Incubation was carried out for 2 h at 37 °C. Toluene-based scintillation fluid was then added directly to the assay vials and ^{14}C-lipids solubilized in the scintillation liquid were counted with high efficiency, as described previously (13). Maximal lipogenesis, obtained by incubation with 10 ng/ml of insulin, was 480% of control values by incubation without added insulin.

Inhibition of Lipolysis—Assay of lipolysis was carried out for 3 h at 37 °C in vials containing 0.5 ml with 3 × 10^5 adipocytes, 0.4 μM isoproterenol, and increasing concentrations of IgG from immune serum. Aliquots of the medium were then taken, bovine serum albumin was removed by trichloroacetic acid precipitation, and the glycerol content was determined by the chemical method which appears in the Pierce Catalogue (Pierce Chemical Co.). The amount of glycerol released was 12 and 140 nm/3 × 10^5 cells/3 h in the absence and presence of 0.4 μM isoproterenol, respectively. Insulin at 10 ng/ml inhibited 87% of the glycerol released.

RESULTS

Insulin-like Receptor Antibodies of the IgG2 Class Develop in Mice Immunized to Insulin—Mice were immunized to insulin, and primary and secondary sera were tested for insulin-like lipogenic activity. Sera obtained up to 10 days after primary immunization had no insulin-like activity. However, insulin-like lipogenic activity was evident in the sera of mice 28, 35, 42, and 56 days after primary immunization (Table I). The insulin-like activity was present whether or not the mice had received a secondary booster immunization to insulin and therefore was independent of either the administration of exogenous insulin or the titer of insulin antibodies in the serum at the time of the assay. Serum obtained from untreated mice had neither insulin antibodies nor lipogenic activity. Fifty per cent maximal lipogenesis was produced by about 0.5 μl of positive late immune serum or 0.15 ng of insulin (not shown). Thus, the secondary serum from immunized mice had peak lipogenic activity that was the equivalent of about 300 ng/ml of insulin.

TABLE I

Insulin antibodies and insulin-like receptor antibodies developing after immunization to insulin

Mice were immunized with insulin on day 0, and some were boosted by a secondary immunization on day 21. Sera were tested for the titer of insulin antibodies and for receptor antibody activity measured as the per cent lipogenic activity.

Serum		Insulin antibodies	Receptor antibodies
Days after primary immunization to insulin	Secondary boost at day 21		
		titer	*% lipogenic activity*
0	None	$<10^{-1}$	0
7	None	10^1	0
28	None	10^1	160
	+	10^4	160
35	None	10^2	150
	+	10^5	140
42	None	10^2	45
	+	10^4	60
56	None	10^1	50
	+	10^3	60

The role of insulin antibodies in producing lipogenic activity was tested by passing the secondary serum through a column of insulin-agarose. The effluent from this column contained all of the lipogenic activity (Fig. 1), but none of the antibodies to insulin. All of the antibodies to insulin bound to the immobilized insulin and could be recovered in the fraction that was eluted from the column (not shown). Hence, the lipogenic activity of the serum from the immunized mice could not be attributed to antibodies to insulin.

To identify the substance with lipogenic activity, we passed the positive serum through a column of protein A linked to Sepharose. Table II shows that the effluent fraction contained all of the insulin antibodies and none of the lipogenic activity, while the fraction of serum eluted by 0.01 M HCl contained all of the lipogenic activity and none of the insulin antibody. As protein A at pH 7.0 binds primarily the IgG2 fraction of mouse antibodies and possibly IgG3 which is a minor fraction of the total IgG (10), it seemed that the lipogenic substance was a property primarily of IgG2 antibodies. This was confirmed by Ouchterlony analysis of the Ig class of the eluted material which was found to be IgG2 (not shown). Mouse antibodies to insulin in the same sera were affinity purified and eluted from an insulin-agarose column and were found to be largely of the IgG1 class (not shown). Hence, the lipogenic activity could be attributed largely to an IgG2 class of insulin-like antibodies that was distinct from the IgG1 class of antibodies to insulin.

Fig. 2 shows the dose-response characteristics of protein A-purified insulin-like IgG2 antibodies. Fifty per cent of the lipogenic activity was produced by a concentration of about 4 μg/ml. Fig. 3 shows that the protein A-purified insulin-like antibodies, similar to insulin (14), could inhibit lipolysis. Fifty per cent of maximal inhibition of lipolysis was produced by about 3 μg/ml.

Insulin-like Receptor Antibodies Displace Receptor-bound Insulin—In an earlier study (1), we observed that serum obtained from mice that had been immunized to insulin could displace insulin from the insulin receptors of adipocytes. To learn whether the displacement of insulin was caused by receptor antibodies or by contaminating insulin antibodies, we removed any residual insulin antibodies by passing the purified IgG2 through an insulin-agarose column. Fig. 4 shows that radiolabeled insulin specifically bound to adipocytes was

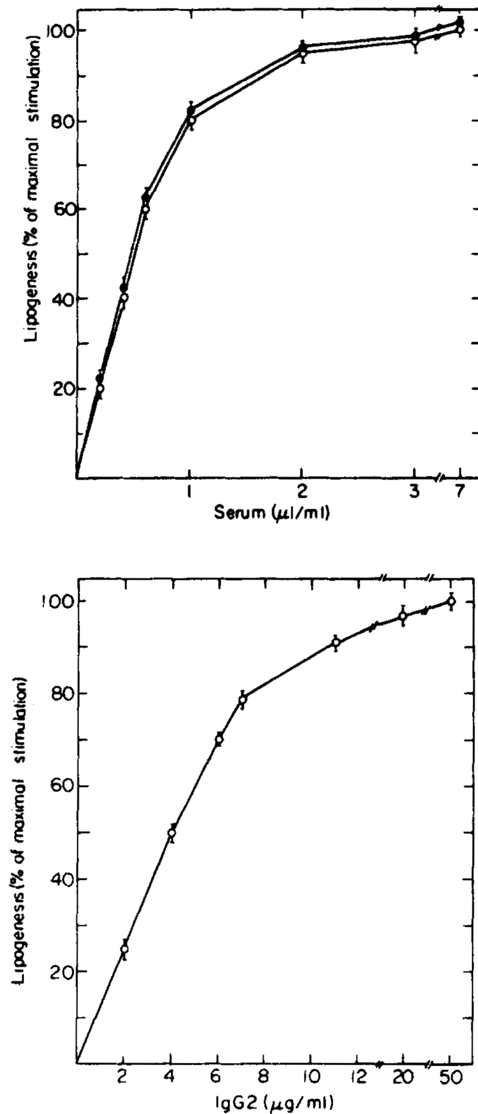

FIG. 1 (*top*). **Stimulation of lipogenesis by secondary serum depleted of insulin antibodies.** Lipogenesis was carried out with increasing concentrations of secondary serum (O) or secondary serum that was depleted of insulin antibodies using an insulin-agarose column (●).

FIG. 2 (*bottom*). **Stimulation of lipogenesis by IgG2 from immune serum.** Lipogenesis was produced by the indicated concentrations of IgG2 that was purified from secondary serum by elution from a column of protein A-Sepharose.

displaced to an equal degree by either the intact IgG2 or the IgG2 fraction depleted of insulin antibodies. Thus, IgG2 insulin-like antibodies free of insulin antibodies appeared able to bind to insulin receptors of adipocytes and displace insulin. This operationally defined the insulin-like antibodies as insulin receptor antibodies.

Lipogenic Activity is Not the Result of Insulin-Antibody Complexes—To investigate whether insulin-antibody complexes could contribute to the lipogenic activity of the receptor antibodies, we incubated radiolabeled insulin with positive serum for 24 h to produce complexes of insulin antibodies and radiolabeled insulin. These complexes were dissociated by lowering the pH to 2.7 and were separated into high and low molecular weight fractions of the serum by gel chromatography using Sephadex G-10. Fig. 5 shows that most of the radiolabeled insulin appeared in the included volume of the column, probably along with any unlabeled insulin present in the serum. The high molecular weight fraction that contained antibodies but no complexed insulin accounted for almost all of the lipogenic activity. Hence, there was no evidence that the lipogenic activity could be attributed to insulin-antibody complexes, a conclusion also supported by the observations that the lipogenic receptor antibodies and insulin antibodies belonged to diverse IgG isotypes, that they could be separated using columns of immobilized insulin (Fig. 1) or Sepharose-protein A (Table II), and that lipogenic activity was independent of the titer of insulin antibodies in the serum (Table I).

Receptor Antibodies Are Anti-idiotypes—Anti-idiotypic antibodies are defined by their interaction with specific idiotypes (3). In an earlier study, we found that the insulin-like receptor antibodies could be bound by immobilized antiserum obtained from guinea pigs that had been immunized to insulin (1).

To prove that the receptor antibodies were spontaneous anti-idiotypes, we undertook to isolate the specific mouse idiotypes with which the receptor antibodies might react. Insulin antibodies were affinity purified from either primary or secondary sera, and the effects of these antibodies on the insulin-like antibodies were tested. Table III shows the results of incubating increasing concentrations of insulin receptor antibody with purified insulin antibodies obtained from 10-day primary or secondary (10 days after boost) serum. It can be seen that the lipogenic activity of the receptor antibodies was strongly inhibited by the 10-day primary insulin antibodies of the immunized mice, but not by the secondary insulin antibodies. Therefore, it appears that the specific idiotype was present only during the early primary response, while the anti-idiotypic receptor antibodies were detectable only during the late primary or secondary responses. Hence, the anti-idiotypic receptor antibodies were observed to coexist with late insulin antibodies of unspecified idiotypes, but not with primary insulin antibodies containing the specific idiotype.

Critical Insulin Epitope Triggers Anti-idiotypic Receptor Antibodies—To gain information about the antigenic determinant, or epitope, of insulin responsible for triggering the specific idiotypic-anti-idiotypic network, we chemically modified the insulin molecule at its three free amino grups (A1, B1, and B29) and compared the biological and immunological functions of the native and modified molecules. The three positions were chosen because two of them (A1 and B1) have been shown to be essential for receptor activity (15). Table IV shows the relative lipogenic activities of three modified

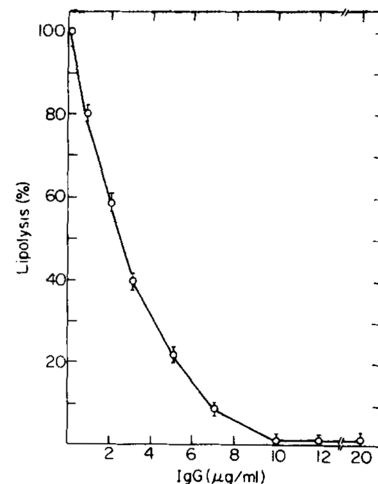

FIG. 3. **Inhibition of lipolysis by IgG2 from secondary serum.** Assay of lipolysis was carried out in the presence of increasing concentrations of IgG2 from secondary serum.

TABLE II

Separation of lipogenic activity from insulin antibodies by affinity chromatography with Sepharose-protein A

Secondary serum was tested whole or fractionated by Sepharose-protein A affinity chromatography. The effluent fraction was collected by washing the column with a Krebs-Ringer buffer containing 2% bovine serum albumin (pH 7.0), and the eluate was collected with 0.01 M HCl and neutralized. Insulin antibodies were measured using a solid phase radioimmunoassay, and the titers were corrected for equal volumes. Lipogenic activity was measured, and the percentage was computed relative to that present in the whole serum.

Treatment of secondary serum	Serum fraction	Insulin antibody titer	Lipogenic activity
			%
None	Whole	10^{-4}	100
Protein A column	Effluent	10^{-4}	<1
	Eluate	$<10^{-}$	80

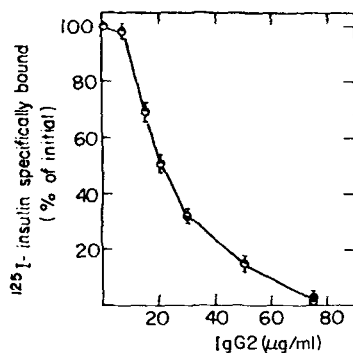

FIG. 4. **Displacement of ^{125}I-insulin from fat cells by IgG2 from secondary serum.** Suspensions of fat cells were incubated with increasing concentrations of IgG2 from secondary serum (○) or IgG2 from secondary serum that was depleted of insulin antibodies, using a column of agarose-insulin (●). The percentage of initial binding is corrected for nonspecific binding that was not displaceable by an excess of unlabeled insulin (15 µg/ml).

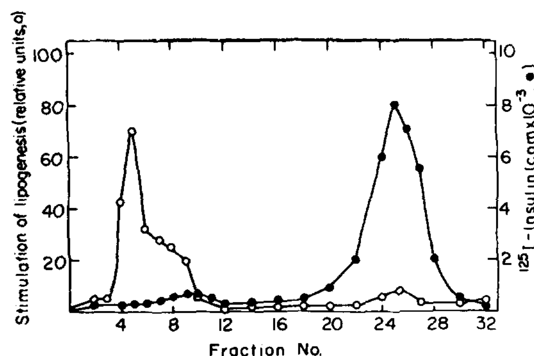

FIG. 5. **Lipogenesis following dissociation and separation of insulin-antibody complexes.** Secondary serum was equilibrated with ^{125}I-labeled insulin, and insulin-antibody complexes were dissociated by treatment with low pH. The insulin was separated from high molecular weight antibodies by gel filtration using Sephadex G-100. The fractions were tested for the presence of labeled insulin and for stimulation of lipogenesis recorded as relative units.

insulins and their immunological potency in inducing insulin antibodies and receptor antibodies following primary and secondary immunization. Acetyl$_3$ insulin, succinyl$_3$, and trinitrobenzene insulins demonstrated 10%, 2%, and less than 1% of the lipogenic activity of insulin, respectively. However, all three modified insulins were immunogenic and stimulated comparable titers of antibodies binding to native insulin. Nevertheless, little or no receptor antibody was generated in response to the modified insulins. These results support the conclusion that induction of receptor antibody is not a function of the gross immune response to insulin, but depends on the fine response to the particular epitope whose conformation involves the amino acid residues responsible for biological activity of insulin.

TABLE III

Inhibition of receptor antibody lipogenesis by primary insulin antibodies

Amounts of insulin receptor antibody (protein A-purified IgG2) were tested for lipogenic activity in the presence or absence of affinity purified insulin antibodies (4 µg/ml) obtained during the primary (day 10) or secondary (day 31; boost on day 21) responses to immunization to insulin.

Affinity purified insulin antibody (4 µg/ml)	Insulin receptor antibody	Lipogenesis[a]	Inhibition of lipogenesis
	µl	%	%
None	1	58	
	2	62	
	4	95	
Secondary	1	60	0
	2	70	0
	4	96	0
Primary	1	7	88
	2	10	86
	4	20	79

[a] Per cent of maximal lipogenesis obtained using 10 ng/ml of insulin.

TABLE IV

Development of receptor antibodies inhibited by alteration of insulin molecule

Mice were immunized with native or modified insulins, and their sera were studied on days 14 and 35 for the titer of antibodies to native insulin and on day 35 for receptor antibody activity. The biological activity of the insulins was computed relative to that of native insulin (100%) from the dose-response curves.

Immunizing insulin	Biological activity	Antibodies to native insulin		Day 35 receptor antibodies
		Day 14	Day 35	
	%	*titer*		*% lipogenic activity*
Native	100	10^3	10^4	150
Acetyl$_3$	10	10^3	10^4	10
Succinyl$_3$	2	10^3	10^4	7
Trinitrobenzene$_3$	<1	10^3	10^4	5

DISCUSSION

Insulin receptor antibodies were operationally defined in this study by their ability to bind to the insulin receptors of adipocytes and to mimic the hormonal functions of insulin, such as lipogenesis and antilipolysis *in vitro*.

Most of the receptor antibodies were found to be members of the IgG2 class of immunoglobulins that bind specifically to staphylococcal protein A (Table II). Antibodies from the same secondary serum binding to insulin, in contrast, were of the IgG1 class. There was no evidence that the insulin-like effects were due to insulin antibodies or insulin-antibody complexes, as dissociation of such complexes (Fig. 5) or removal of insulin antibodies from serum did not affect activity (Fig. 1).

In a previous study, we found that the receptor antibodies interacted with insulin antibodies of immunized guinea pigs, indicating that the receptor antibodies were also anti-idiotypes to insulin antibody idiotypes (1). Thus, the insulin receptor antibodies could have arisen as products of an idiotypic-anti-idiotypic network set in motion by immunization to an epitope of insulin. The results of the experiments

reported here confirmed this hypothesis by demonstrating the appearance in the mouse of the specific idiotype and the critial epitope of the insulin molecule.

According to the idiotypic-anti-idiotypic network hypothesis, the binding site of the anti-idiotypic antibody could have a conformation similar to that of an epitope on the original antigen (3, 4). This is because the binding site of the idiotypic antibody (to which the anti-idiotype is directed) is complementary in structure to the epitope. Therefore, the critical insulin epitope in our case should be that portion of the insulin molecule recognized by the insulin receptor; thus, the anti-idiotype binds to the insulin receptor because it looks like the functional part of insulin.

Chemical modification of the NH_2-terminal amino groups of positions A1 and B1 has been shown to inhibit the binding of the modified insulin to its receptor (reviewed in Ref. 15) and, as illustrated in Table IV, to considerably affect its biological activity. These effects can best be explained by disruption of the structure of the molecule necessary for its binding to the receptor. The fact that modified, biologically inactivated insulin could trigger the production of high titers of antibodies that recognized native insulin indicates that a considerable amount of native structure remained intact despite modification and suggests that the modification was selective for the binding region of the hormone. The specific idiotype was probably limited to B lymphocytes and their antibodies, as T lymphocytes have been found to recognize primarily the part of the insulin molecule containing the A chain loop (16). In any case, it is significant that a chemical alteration abolished both the receptor activity of the insulin and the generation of receptor antibodies without decreasing the gross titer of native insulin antibodies following immunization (Table IV). Hence, the epitope important for receptor binding was critical, as predicted, for inducing the specific anti-idiotypic receptor antibody, presumably by way of the specific idiotype.

Jerne (3) proposed that anti-idiotypic antibodies function to regulate immune responses by interacting with specific antibodies, and it has been shown that anti-idiotypes can suppress or enhance the development of idiotypes (17-19). Suppression of the specific primary idiotype by the IgG2 anti-idiotypic receptor antibodies could explain the restriction of the specific idiotype to the primary response and the failure to detect the idiotype and the anti-idiotype in the same serum. It has been shown that anti-idiotypic antibodies of the IgG2 class in guinea pigs are particularly suppressive of idiotypes (19) and might function similarly in mice.

That anti-idiotypes can act as insulin-like antibodies was shown by Sege and Peterson (20), who immunized rabbits with purified insulin antibodies of rats. The rabbits produced anti-idiotypic antibodies, some of which could interact with the insulin receptor. An anti-idiotype that acts as an antibody to the β-adrenergic receptor has also been described by Schreiber *et al.* (21).

Beyond these theoretical considerations, it is clear that immunization to insulin can lead to the development of insulin-like receptor antibodies. It is shown in the accompanying article that the development of insulin-like receptor antibodies in mice has profound consequences for glucose homeostasis *in vivo*. This suggests that the receptor antibodies are physiologically significant and not merely an experimental aberration. This observation has implications for the many diabetic persons who, treated with insulin, produce insulin antibodies. Might not some of them produce receptor antibodies (22)?

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