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Mice Immunized to Insulin Develop Antibody to the Insulin Receptor

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We immunized mice with insulin and found that those strains that develop insulin antibodies subsequently produce insulin-like activity in amount equivalent to 300–400 ng insulin per ml serum. The activity was due exclusively to IgG2 antibodies. Bioactivity could be blocked efficiently by insulin antibodies from guinea pigs and from mice. The active IgG2 also displaced labeled insulin from fat cells. Preliminary in vivo studies have indicated that the appearance of insulin-like antibodies in the mouse resulted in abnormal glucose homeostasis and "down regulation" of insulin receptors. These results indicate that immunization to insulin can initiate an idiotype-anti-idiotype network resulting in antibodies to the hormone receptor.

Key words: autoantibodies, insulin receptor, anti-idiotypes, insulin-immunized mice

Several diseases of man can be related to the formation of autoantibodies that interact with surface receptors. Examples are the antibodies that trigger TSH receptor in Graves disease [1], antibodies that bind to and block the receptor for acetylcholine at the neuromuscular junction in myasthenia gravis [2], and antibodies that trigger insulin-like responses appear in certain (very rare) types of severe insulin-resistant diabetes [3].

Sege and Peterson [4] and Jerne [5] raised possible explanations for receptor autoimmunity. According to their hypothesis, the immune system may be regulated by a network in which antigen (such as hormone) induces production of idiotypes which in turn induce anti-idiotypes that can feed back to shut off or modify the original idiotypic response. Some of the anti-idiotypes against the hormone-binding region of the idiotypes may mimic the structure of the hormone itself. Such anti-idiotypic antibodies might bind to the hormone receptor and thus function as antibodies to the receptor. Accordingly, Sege and Peterson [4] immunized rabbits against affinity-purified insulin antibodies, and obtained anti-idiotypic antibodies that bound to the insulin receptor.

In the present study we demonstrate that immunization of mice to ungulate insulins can lead to the development not only of antibodies to insulin, but of insulin-like antibodies (ILA) that appear to recognize the insulin receptor and to trigger the

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bioresponses of insulin at low concentrations. The characteristics and the properties of these antibodies are summarized.

MATERIALS AND METHODS

Animals

Female mice of the strains (C3H/ebxC57BL/6)F1 hybrid, BALB.C3H, C3H.SW, and BALB/c and male Wistar rats (70–100 g) originated from the Animal Breeding Center of the Weizmann Institute.

Materials

D(U-^{14}C)glucose (4–7 mCi/mol) was purchased from New England Nuclear, collagenase type I (134 U/mg) from Worthington, protein A–Sepharose column from Pharmacia. Insulin-agarose was prepared as described [6].

Methods and Procedures

The following procedures were used with no modification: Immunization of mice to insulin [7], a solid-phase radioimmunoassay to insulin antibodies [8], SDS-mercaptoethanol gel electrophoresis [9], affinity purification of insulin antibodies on an insulin-agarose column [10], separation of IgG from immune serum by protein A–Sepharose column [10], Ouchterlony assay of precipitation in agar for determining antibody class [11], preparation of isolated fat cells from epididimal fat pads [12], binding of ^{125}I-insulin to fat cells [13], lipogenesis [14], and inhibition of lipolysis [10].

RESULTS

Occurrence and Appearance of Insulin-Like Activity in the Mice

The immunization of mice with ungulate insulin resulted in the appearance of insulin antibodies initially detected 7–10 days after the primary injection. A second injection of insulin at 3 weeks was followed by the appearance of insulin-like activity initially detected at days 9–11 after the booster injection (in preparation). The insulin-like activity persisted for 3–5 days and then declined gradually and disappeared over a period of 5 days. A second peak of insulin-like activity was evident 30–35 days after the booster injection (in preparation). Control serum obtained from mice not immunized to insulin showed no detectable amount of insulin antibodies and no insulin-like activity (not shown). Mice genetically incapable of mounting an immune response to ungulate insulins also did not produce insulin-like activity. For example Table I shows that C3H.SW strain (H-2^b) mice produced both insulin antibodies and insulin-like activity in response to immunization with bovine insulin but not with sheep or porcine insulin, whereas BALB.C3H (H-2^k) mice responded to sheep insulin and BALB/c (H-2^d) responded to all three insulins.

Identification of the Serum Fraction With Insulin-Like Activity

Figure 1 demonstrates fractionation of an immune serum on protein A–Sepharose affinity column. We loaded an immune serum obtained 12–14 days after booster injection (see previous section). This serum contained both insulin antibodies in high titer (10^4 dilution^{-1}) and insulin-like activity detectable at 0.4–1 $\times$ 10^4 serum dilu-

TABLE I. Production of Insulin Antibodies and ILA by Different Mouse Strains

Mouse strain	H-2	Immunized with insulin from	Insulin antibody titer[a] (dilution^{-1})	ILA[b] (fold dilution for obtaining 50% of maximal lipogenesis)
BALB.C3H	k	Bovine	< 10	10
		Sheep	10^3	10^3
		Porcine	< 10	10
C3H.SW	b	Bovine	10^3	10^3
		Sheep	< 10	10
		Porcine	< 10	10
BALB/c	d	Bovine	10^3-10^4	10^3
		Sheep	10^3-10^4	10^3
		Porcine	10^3-10^4	10^3

[a]Insulin antibody titer was determined on day 8 after the booster injection (see text).
[b]The ability of the serum to stimulate lipogenesis was tested 12 days after booster injection (see text).

tion^{-1}. We found that the fraction containing the insulin-like activity was quantitatively adsorbed to the column at pH 7.0 and could be eluted at a lower pH (pH 3.0). The fraction that did not bind at pH 7.0 contained the insulin antibodies (Fig. 1). Since protein A binds specifically to the Fc portion of antibodies, primarily of the IgG2 class at pH 7.0 [15], it seemed that the insulin-like activity was a property of IgG2 molecules. This was further confirmed by means of polyacrylamide gel electrophoresis, which demonstrates the complete absence of detectable protein band(s) other than immunoglobulin G (Fig. 2). Ouchterlony precipitation of affinity-purified insulin antibody indicated that these antibodies were primarily IgG1 (summarized). As mouse IgG1 is not adsorbed to protein A–Sepharose at pH 7.0 [16], this one step of purification was sufficient to separate insulin antibodies (IgG1) from the insulin-like activity (IgG2).

Overall Properties of Protein A–Sepharose–Purified ILA

Tables II, III, and IV summarize the characterization and properties of the purified ILA fraction, part of which has appeared recently in the literature [10]. Briefly, ILA is an immunoglobulin IgG2 class that does not contain free insulin, antibodies to insulin, or insulin-anti-insulin immune complexes. This IgG2 fraction produces insulin-like bioactivities at concentrations in the microgram range (Table III). In vitro bioactivities are blocked efficiently by affinity-purified guinea pig insulin antibodies or by mouse affinity-purified insulin antibodies (idiotypes) isolated at days 7–10 after the primary injection but not at later periods (Table III and manuscript in preparation). ILA also displace labeled insulin from fat cells, indicating that those antibodies are directed to the region of the insulin receptor that binds insulin. The insulin-like activity of an immune serum emerged quantitatively in the void volume of a Sephadex G-100 column under conditions that dissociate immune complexes (Table IV). Therefore, ILA activity could not be explained by insulin, contaminating or complexed with the IgG2 antibodies.

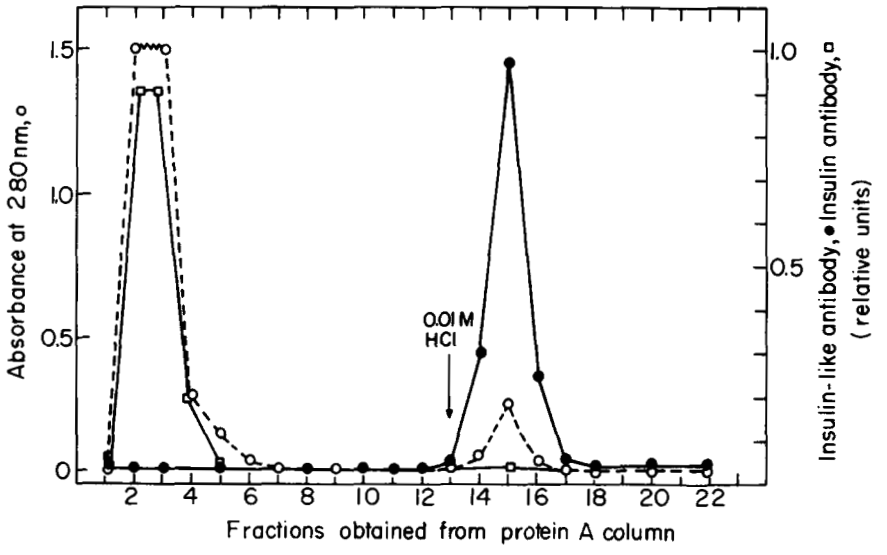

Fig. 1. Purification of IgG2 from immune serum on protein A-Sepharose column (Pharmacia 2.0 × 0.5cm) that was preequilibrated and washed with 0.05 M sodium phosphate (pH 7.0). Fractions of 0.4 ml were collected. The eluted fractions were collected into tubes containing a sufficient amount of $NaHCO_3$ to neutralize the acidity. The fractions were examined for their absorbance at 280 mm (○), ILA (●), and insulin antibodies (□).

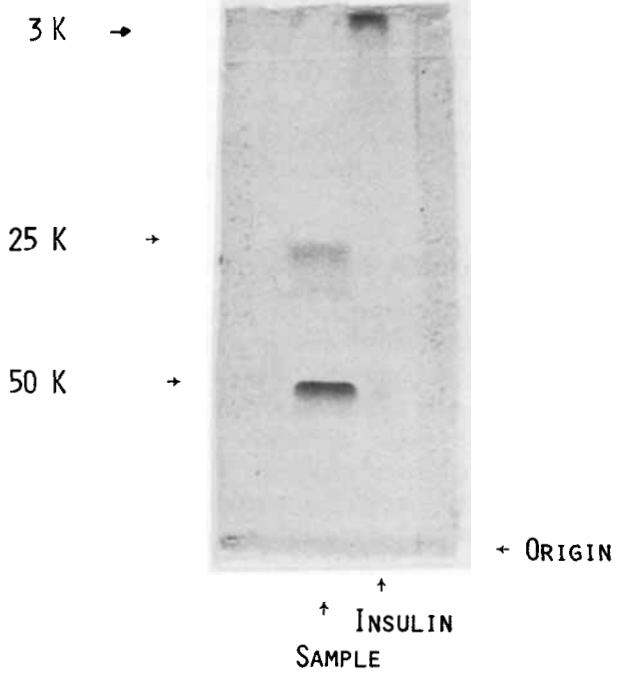

Fig. 2. SDS-polyacrylamide gel electrophoresis of IgG2 isolated from immune mouse serum by protein A-Sepharose column (left lane); insulin (right lane).

TABLE II. Characterization and Properties of ILA, Isolated From Immune Mouse Serum by Protein A–Sepharose Column*

Property	As judged by
Pure IgG	SDS-polyacrylamide gel electrophoresis
IgG2 antibodies	Ouchterlony analysis
Does not contain insulin antibody	Solid-phase RIA[a]
Stimulates lipogenesis (ED_{50} = 4 μg ml^{-1})	Assay of lipogenesis in fat cells
Inhibits lipolysis (ID_{50} = 3 μg ml^{-1})	Assay of lipolysis
Activity inhibited by insulin antibody	Affinity-purified insulin antibodies added to lipogenesis assay
Displaces insulin from receptor	Competes with the binding of labeled insulin to fat cells (50% displacement at 20 μg ml^{-1})

*Details of the isolation of IgG2 from immune serum are given in the legend to Figure 1.
[a]Insulin antibodies from the mouse were found to be of the IgG1 class (by Ouchterlony analysis). This type is not adsorbed to the agarose-protein A column under the conditions applied here.
The void volume contained all ILA activity but not insulin.

The development of ILA in mice has profound consequences for glucose homeostatis in vivo (in preparation). The most prominent symptoms are gradual increase in fasting blood glucose level, and diabetic glucose tolerance tests. It seems likely that those disturbances in glucose homeostasis are in part the consequence of decreased number of insulin receptors in those mice (in preparation).

DISCUSSION

The present study indicates that mice that develop antibodies to ungulate insulins also develop insulin-like antibody (ILA) that binds to the insulin receptor of fat cells and can mimic the hormonal functions of insulin both in vitro and in vivo (in preparation). These antibodies, which were identified to belong to the IgG2 class, seem to be anti-anti-insulin antibodies (anti-idiotypes) based on the ability of affinity-purified mouse insulin antibodies to block their bioactivities (Table II).

Since ILA binds to the insulin receptor in fat cells, it seems logical to assume that ILA may be complementary to those insulin antibodies that bound to the part of insulin seen by the insulin receptor. The restriction of idiotype-specific insulin antibodies to the primary antibody response to insulin may be explained by the presence of anti-idiotypic IgG2 ILA that may have suppressed this idiotype in the secondary response. It was previously found that antiidiotypic antibodies of the IgG2 class were particularly suppressive of the idiotype [17].

One of the more important questions is why anti-idiotypic antibodies are produced in the immunized mice. The insulin molecule of mice has been conserved in the mainstream of insulin evolution in mammals and differs from pork and beef insulins by three and five amino acid substitutions [18] that are structurally confined to the variable domain of the molecule that does not interact with insulin receptor [19,20]. In response to immunization of the mouse with ungulate insulins, the mouse

TABLE III. Lipogenesis Produced by Immune IgG2. Inhibition by Idiotypic Insulin Antibodies

Addition	μg/ml	Incorporation of (U-^{14}C)glucose into lipids cpm/1.5 × 10^4 cells/h	% Maximal stimulation
None		1,400	0
Insulin (10 ng/ml)		7,200	100
Normal IgG2	20	1,400	0
Immune IgG2	0.1	1,400	0
	1	1,980	10
	2	2,750	23
	3	3,800	41
	4	4,700	57
	6	5,450	70
	8	6,200	83
	10	6,600	90
	20	6,900	95
	50	7,200	100
Immune IgG2 (8 μg/ml) + idiotype (20 μg/ml)[a]		1,800	7

[a]Isolated by insulin-agarose column 7–10 days after the primary injection.

TABLE IV. Lipogenesis Produced by Receptor Antibody Dissociated From Insulin

Serum equilibrated with ^{125}I-insulin[a]	Dissociation and fractionation fraction	^{125}I-insulin (cpm)	Lipogenesis (cpm)
Control	Void	700	600
	Included	70,000	700
Immune	Void	800	6,000
	Included	67,000	1,000

[a]Immune or control serum (0.2 ml) was equilibrated with labeled insulin (100,000 cpm) for 24 h at 4°C. The serum was then acidified to pH 2.7 and loaded on a Sephadex G-100 column (40 × 0.8 cm) that was preequilibrated and washed with 0.1 M acetic acid–0.2% BSA (pH 2.7). The fractions corresponding to the void volume (identified by dextran blue) or those corresponding to the included volume (identified by their radioactive content) were pooled and neutralized with $NaHCO_3$. Aliquots were withdrawn to determine their radioactive content, and for activation of lipogenesis.

produces antibodies that recognize the hormonal domain of the molecule that is common to most mammalian insulins [18] and to mouse insulin itself. Hence, in response to ungulate insulin, the mouse probably makes autoantibodies. It is tempting to speculate that the mouse makes anti-idiotypic antibodies to regulate its autoantibodies to the hormonal domain of insulin. The price of these anti-idiotypes is their ILA activity at the insulin receptor.

Going along with this line of reasoning, guinea pig insulin, in contrast, is an evolutionary deviation in the mammalian kingdom and differs from pork insulin in 17 of its 51 positions [18] and probably has a unique structure in its hormonal domain [21,22]. Hence, in response to ungulate insulin the guinea pig can make antibodies to the same antigenic determinants on the pork insulin as does the mouse. However, as guinea pig insulin differs radically from ungulate insulin, these antibodies may not be

autoantibodies to its own insulin [21]. The guinea pig is not constrained to regulate the idiotype which for him is not an autoantibody. On the other hand, the chicken, although belonging to a different order, has insulin molecules that differ by only four amino acid substitutions from pork or beef insulin [22] and is either similar to or more bioactive than mammalian insulins [22]. Hence, immune chicken is expected to produce ILA as does the mouse.

Indeed preliminary experiments indicate that guinea pig immunized to insulin produces a relatively high concentration of idiotype and no ILA activity, whereas chicken produces low concentrations of idiotype following the production of ILA (in preparation). This implies that anti-idiotypic network envisioned by Jerne [5] might be especially apt to function to regulate autoimmune reactions. In summary, immunization to insulin can lead to the development of ILA. The study has many implications and can be extended in various directions. It evokes questions such as 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? These and related questions are under study.

REFERENCES

1. Hearn MTW: Trends Biochem Sci 5:75, 1980.
2. Fuchs S: Curr Top Microbiol Immunol 85:1, 1979.
3. Flier JS, Kahn CR, Jarett DB, Roth J: J Clin Invest 58:1442, 1976.
4. Sege K, Peterson PA: Proc Natl Acad Sci USA 75:2445, 1978.
5. Jerne NK: Ann Immunol Pasteur 125C:373, 1974.
6. Cuatrecasas P: Proc Natl Acad Sci USA 69:1277, 1972.
7. Cohen IR, Talmon J: Eur J Immunol 10:284, 1980.
8. Eshhar Z, Strassmann G, Waks T, Mozes E: Cell Immunol 47:378, 1979.
9. Laemmli UK: Nature 227:680, 1970.
10. Shechter Y, Maron R, Elias D, Cohen IR: Science 216:542, 1982.
11. Ouchterlony O: Prog Allergy 5:1, 1958.
12. Rodbell M: J Biol Chem 239:375, 1964.
13. Cuatrecasas P: Proc Natl Acad Sci USA 68:1264, 1971.
14. Moody AJ, Stan MA, Stan M, Gliemann J: Horm Metab Res 6:12, 1974.
15. Kessler SW: J Immunol 115:1617, 1975.
16. Ey PL, Prowse SJ, Jenkin CR: Immunochemistry 15:429, 1978.
17. Eichmann K, Rajewsky K: Eur J Immunol 5:661, 1975.
18. Dayhoff MO (ed): "Atlas of Protein Sequence and Structure," D186. Washington DC: National Biomedical Research Foundation.
19. De Meyts P, Van Obberghen E, Roth J, Wollmer A, Brandenburg D: Nature 273:504, 1978.
20. Ranghino G, Talmon J, Yonath A, Cohen IR: In: "Structural Aspects of Recognition and Assembly in Biological Macromolecules." Philadelphia: Balaban ISS, 1981, p 263.
21. Neville RWJ, Weir BJ, Lazarus NR: Diabetes 22:851, 1973.
22. Blundell T, Dodson G, Hodgkin D, Mercola D: Adv Protein Chem 26:279, 1972.