immunizations with cells bearing SP receptors at their surface. Moreover, the neurokinin A (substance K) receptor has recently been cloned,[25] using methodology that also does not require purified receptor. No doubt the receptor for SP will also be cloned in the near future, and this will allow a large variety of antibodies to SP receptor or its fragments to be raised. However, in our opinion, the interest of the anti-idiotypic approach lies in the production of antibodies directed in principle against the receptor binding site in its native conformation, i.e., antibodies pharmacologically active *in vivo* and thus of possible value as therapeutic agents in specific pathologies.

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[25] Y. Masu, K. Nakayama, H. Tamaka, Y. Harada, M. Kuno, and S. Nakanishi, *Nature (London)* **329**, 836 (1987).

[20] Production and Properties of Anti-idiotypic Antibodies That Recognize Insulin Receptor

By IRUN R. COHEN, DANA ELIAS, MICHA RAPOPORT, and YORAM SHECHTER

Introduction

The immune system and the endocrine system both process information by ligand–receptor interactions. In the endocrine system, the ligands are the hormones and the receptors are the specific hormone receptors. The amino acid sequences and hence the structures of those molecules are fixed by the germ line genome. In the immune system, the ligands are the epitopes of antigens and the receptors are the antigen receptors of T or B cells, or the antibodies produced by the latter. The structures of these molecules vary as a consequence of somatic gene recombination (the

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receptors) and immunogenic encounters (the antigens). Because of the vast numbers of potential antigen receptors it is difficult to imagine a biological structure that cannot be recognized by virtue of its complementarity to at least some antigen receptors. Hence, there is an immense number of potential antigens. Consequently the binding portions of antigen receptors themselves may be seen as unique antigens (idiotypes) by anti-idiotypic receptors of other lymphocytes.[1]

In the special case where an idiotypic antibody and its complementary anti-idiotypic antibody resemble a hormone or a hormone receptor, the immune system may be induced to produce antibodies that can interact with hormones and hormone receptors. The consequences of such antibodies, produced without endocrine control, may play havoc with the body's homeostasis. Our studies relating to this circumstance involve the production and properties of antibodies that mimic the structure of the hormone insulin, specifically that part of the hormone that fits the hormone receptor. Such insulin-mimicking antibodies can interact, like insulin, with the insulin receptor and function as antireceptor antibodies.

The development of an insulin-mimicking antibody arising as an anti-idiotypic antibody was first contrived by Sege and Peterson.[2] They immunized rats with insulin, isolated the rat antiinsulin antibodies, and then used the antibodies to immunize rabbits. A few of the rabbits produced antiantiinsulin antibodies (anti-idiotypes), some of which were observed to mimic insulin. Our extension of the Sege–Peterson work was based on the discovery that mice immunized to ungulate insulin spontaneously produced insulin-mimicking anti-idiotypes that functioned as antireceptor antibodies.[3] These anti-idiotypes followed and replaced idiotypic antibodies that recognized as their epitope the part of the insulin molecule interacting with the hormone receptor.[4,5] Thus, the preceding idiotype, which we now term the DM idiotype (DM Id), mimicked the insulin binding site of the hormone receptor. It is reasonable to postulate that the receptor-mimicking DM Id triggered, by a network interaction,[1] the insulin-mimicking anti-DM anti-idiotype (anti-DM Id).

In this chapter, we describe how we have raised DM Ids and anti-DM Ids as monoclonal reagents. The properties of the anti-DM Id antibodies and their effects on the health of animals and humans are briefly summarized.

[1] N. K. Jerne, *Ann. Immunol.* (*Paris*) **125C,** 373 (1974).
[2] K. Sege and P. A. Peterson, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2443 (1978).
[3] Y. Shechter, R. Maron, D. Elias, and I. R. Cohen, *Science* **216,** 542 (1982).
[4] Y. Shechter, D. Elias, R. Maron, and I. R. Cohen, *J. Biol. Chem.* **259,** 6411 (1984).
[5] D. Elias, R. Maron, I. R. Cohen, and Y. Shechter, *J. Biol. Chem.* **259,** 6416 (1984).

Materials and Methods

Animals. Female mice of the (BALB/c × C57BL/6) F_1 hybrid strain (Jackson Laboratories, Bar Harbor, ME) were immunized between 2 and 4 months of age. Male Wistar rats (70–100 g) used as source of adipocytes originated from the colony of the Department of Hormone Research, Weizmann Institute (Rehovot, Israel). Mice were immunized by inoculation into each hind footpad of 25 μg of bovine insulin emulsified in Freund's complete adjuvant.[4]

Reagents. D-[U-^{14}C]Glucose (4–7 mCi/mol) was purchased from New England Nuclear; collagenase type I (134 units/mg) was bought from Millipore Corp. (Worthington, NJ); bovine insulin and bovine serum albumin were purchased from Sigma (St. Louis, MO); guinea pig antiinsulin antiserum was from Bio Yeda (Rehovot, Israel); and ^{125}I-labeled goat anti-mouse immunoglobulin was from Amersham (Buckinghamshire, UK).

Lipogenesis. Lipogenesis was performed as described.[4] The ability of anti-idiotypes to stimulate lipogenesis was tested by the addition of 1–50 μg/ml of monoclonal anti-idiotypic IgG.

Displacement of ^{125}I-Labeled Insulin. The ability of purified anti-idiotypic antibodies to displace ^{125}I-labeled insulin was performed as described.[4] IgG was added at 10–100 μg/ml.

Inhibition of Anti-DM Idiotype Lipogenesis. Anti-idiotypic antibodies, 20 μg/ml, were incubated with adipocytes in a lipogenesis assay. DM Id monoclonal antibodies, 50 μg/ml, were added to the assay, and the reduction in lipogenesis was calculated.

Isotyping of Monoclonal Antibodies. An Ouchterlony assay of precipitation in agar[6] was used to identify the immunoglobulin class of the monoclonal antibodies. Antisera to mouse IgG_1, IgG_2, IgG_{2a}, IgG_{2b}, IgA, and IgM were purchased from Meloy Lab. Inc. (Springfield, VA).

Purification of Monoclonal Antibodies. Monoclonal antibodies were purified from tissue culture supernatants and ascites fluid using a method described by McKinney and Parkinson.[7]

Solid-Phase Radioimmunoassay

1. Binding to insulin: Bovine insulin, 50 μg/ml in PBS, was added to microtiter flexible poly(vinyl chloride) plates (Dynatech, Alexandria, VA) for 2 hr at 26°. Unbound insulin was washed with PBS, and nonspecific binding was blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at 26°. Sera were diluted in 0.1% BSA in PBS, and 25 μl/well was incubated, in duplicate, for 2 hr, 26°. Plates were

[6] O. Ouchterlony, *Prog. Allergy* **5**, 1 (1958).
[7] M. M. McKinney and A. Parkinson, *J. Immunol. Methods* **96**, 271 (1987).

TABLE I
FUNDAMENTALS FACILITATING ISOLATION OF DM Id AND ANTI-DM Id ANTIBODIES

Antibody	Mimicry	Produced by species	Time of appearance after primary immunization of mice (days)	Screening assay
DM Id	Insulin receptor	Mice, guinea pigs	7–12	Neutralization of anti-DM Id activity
Anti-DM Id	Insulin	Mice	24–40	Lipogenesis (fat cells); binding to DM Id (guinea pig); binding competition with insulin

washed 3 times with 0.1% BSA–PBS, and 10^5 cpm of ^{125}I-labeled goat anti-mouse immunoglobulin was added in each well for 2 hr at 26°. The plates were then washed 4 times with 0.1% BSA–PBS, dried thoroughly, and the wells were cut out and counted in a gamma counter.

2. Binding to DM idiotype: Antisera of guinea pigs, immunized to bovine insulin, were used as a source of idiotypes for screening of anti-idiotypes. The serum insulin antibodies were absorbed on insulin and added to the microtiter plates at 1 : 100 dilution for 16 hr at 4°. The rest was as above.

Production of Anti-DM Id Monoclonal Antibodies

Two factors were critical in facilitating isolation of monoclonal anti-DM Ids: timing, knowing when to fuse the anti-DM Id B cells to hybridoma cells; and screening, assaying conveniently antibody reactivity to both the DM Id and the insulin receptor (Table I).

The original discovery of antiinsulin receptor activity was based on investigating polyclonal antibodies for biochemical effects associated with insulin; in most studies we measured lipogenesis, the incorporation into lipids of ^{14}C-labeled glucose.[8] In other words, rather than using standard immunological assays based on precipitation, fluorescence, or antigen binding, we identified insulin-mimicking antibodies by their insulin-like effects on fat cells *in vitro*. The standard assay of lipogenesis is relatively easy to perform, and the signal amplification inherent in a hormone-activated metabolic reaction provided us with a sensitive readout

[8] A. J. Moody, M. A. Stan, M. Stan, and J. Gliemann, *Horm. Metab. Res.* **6**, 12 (1974).

for the very antireceptor antibody we sought.[4] Polyclonal antibodies screened for insulinlike effects on fat cells were then confirmed as antireceptor antibodies by their competition with insulin for binding to insulin receptors.[4] Anti-DM Id antibodies were found in the sera of mice beginning about 24 days after immunization to bovine or porcine insulin.[4]

The antiinsulin antibodies with the specific DM idiotype were identified by their ability to neutralize the lipogenic effects of the antireceptor, anti-DM Id antibodies.[9] The specific DM Id was found in sera 7–12 days after immunization of mice to insulin, but never thereafter.[9] In contrast to mice, guinea pigs hyperimmunized to ungulate insulins persisted in producing DM Id, and they did not spontaneously produce anti-DM Id.[9] The conclusion that the DM epitope was formed by the receptor binding part of the insulin molecule was derived from investigation of chemically modified or substituted insulins that do not interact with standard mammalian insulin receptors. $Acetyl_3$ insulin, desoctapeptide insulin, or guinea pig insulin do not trigger DM Id or anti-DM Ids,[9] although these insulins can induce antibodies that recognized other epitopes on native unmodified insulins. Because guinea pig insulin differs so markedly from standard mammalian insulin, DM Id antibodies in the guinea pig probably are not autoantibodies; they do not recognize the guinea pig's own insulin. This may be the reason that the guinea pig, in contrast to the mouse, does not produce an anti-DM Id spontaneously.[9]

With this information the isolation of DM Id and anti-DM Id monoclonal antibodies was relatively uncomplicated. We immunized two $2\frac{1}{2}$-month-old female mice of the (BALB/c × C57BL/6) F_1 hybrid strain with a single injection of bovine insulin (Sigma) as described above. Twenty-one days later, several days before the anticipated raise in antireceptor, anti-DM Id antibodies, we collected splenocytes of the mice and fused them using a standard procedure with HAT-sensitive SP2/0 myeloma cells.[10] The mixture of cells was seeded into 960 microtiter wells and grown for 14 days in the presence of HAT medium. The HAT medium was replaced with HT medium, and wells showing cell growth were assayed. A sample of medium (0.05 ml) was removed and tested in a solid-phase radioimmunoassay for antibodies binding either to bovine insulin or to guinea pig antiinsulin antiserum.

The guinea pig antiinsulin antiserum was obtained commercially from Bio-Yeda and found to be rich in DM Id as shown by its neutralization of

[9] Y. Shechter, D. Elias, R. Bruck, R. Maron, and I. R. Cohen, *in* "Anti-Idiotypes, Receptors and Molecular Mimicry" (D. S. Linthicum and N. R. Farid, eds.), p. 73. Springer-Verlag, Berlin and New York, 1988.

[10] Z. Eshhar, *in* "Hybridoma Technology in the Biosciences and Medicine" (T. A. Springer, ed.), p. 3. Plenum, New York, 1985.

TABLE II
ANTI-DM Id ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES

Monoclonal antibody designation	Ig isotype	Effect *in vitro* on insulin receptor	Displacement of ^{125}I-labeled insulin (%)	Lipogenesis (%)	Binding to guinea pig DM Id (cpm)
DT211	IgG_{2b}	Antagonist	60	0	25,810
DT275	IgM	Agonist	50	45	15,000
DT312	IgG	Agonist	65	77	17,250
DT315	IgM	Agonist	70	69	20,500
DT271	IgG_{2b}	Agonist	60	56	15,750
Control	IgG	—	0	0	2,000

the lipogenic, insulin-mimicking activity of anti-DM Id polyclonal antibodies.[9] We used the guinea pig antiserum to screen for three practical reasons: (1) It was readily available in uniform batches. (2) It contained the DM Id along with other, DM-negative antiinsulin antibodies so that we might detect other anti-idiotypes as well as anti-DM Ids. (3) The iodinated goat anti-mouse immunoglobulin antibody used to assay the mouse anti-DM Id did not itself bind strongly to the guinea pig antibodies which were the antigen. What little nonspecific binding was present was easily neutralized by adding 1% normal guinea pig serum to the radioimmunoassay buffer.

Forty-five of the hybridoma culture wells showed binding activity to insulin (40 wells) or to the guinea pig antiinsulin antiserum (5 wells). The latter were expanded and then cloned and recloned by limiting dilution. Table II shows the properties of the 5 monoclonal antibodies that bound to guinea pig antiinsulin. Note that they include both IgM and IgG isotypes. All 5 antibodies competed with insulin for binding to the insulin receptor, indicating that they were able to recognize the insulin receptor. Four out of the 5 mimicked insulin in activating lipogenesis in fat cells and can be considered insulin agonists. The one antibody that competed with insulin for receptor binding but did not activate lipogenesis can be considered an insulin antagonist.

Note that all of the monoclonal antibodies binding to guinea pig antiinsulin antiserum also recognized the insulin receptor. Therefore, they all were anti-DM Ids. Unless there was a sampling error, quite possible with small numbers, this suggests that many if not most of the anti-idiotypes made spontaneously were to the DM Id (the insulin receptor-mimicking antibody). In fact, the only way we have succeeded in raising an anti-idiotypic antibody to antiinsulin *not* of the DM Id has been to artificially

immunize mice with purified DM-negative antiinsulin antibodies in adjuvant, similar to what was done by Sege and Peterson.[2] Thus, mice are capable of making anti-DM-negative anti-Ids, but our experience thus far leads us to suspect that the DM Id uniquely activates an anti-idiotypic network.

Production of DM Id Monoclonal Antibodies

Once we isolated the anti-DM Ids as a set of monoclonals it was relatively easy to select DM Ids as monoclonal antibodies. We merely immunized (BALB/c × C57BL/6) F_1 mice to insulin by injecting them with bovine insulin in Freund's complete adjuvant as before. But, rather than waiting 21 days for the anti-DM Id, we removed the spleens and fused the splenocytes to the myeloma cells 4 days later, 2–3 days before the primary peak of antiinsulin antibodies shown earlier to contain the DM Id.[4]

We screened the culture wells for antibodies binding to bovine insulin using a solid-phase radioimmunoassay (Table III). As expected so early in a primary response, only 4 of the 960 wells were positive for antibodies to insulin. These few cultures could be expanded and cloned without the practical need to detect the DM Id as a precondition for investing the effort. After recloning, we tested the 4 antiinsulin antibodies for their capacity to interact with the monoclonal anti-DM Ids, the simplest assay being neutralization of the lipogenic, insulin receptor activity of the anti-DM Ids. Two monoclonals were DM-positive (DM-ID7 and DM-ID6) and two were DM-negative Ids (DM-ID4 and DM-ID5). The DM-negative antiinsulin antibodies were used to immunize mice to obtain monoclonal anti-idiotypic antibodies that were anti-DM-negative Ids.

TABLE III
DM Id MONOCLONAL ANTIBODIES

Monoclonal antibody designation	Ig isotype	Binding to insulin (cpm)	Inhibition of anti-DM Id lipogenesis (%)
DM-ID7	IgG_1	20,500	85
DM-ID6	IgM	25,300	60
DM-ID4	IgG_1	35,000	2
DM-ID5	IgG_3	24,500	0
Control	IgG	2,000	0

Biological Properties of Anti-DM Id

Because anti-DM Id interacts with the insulin receptor, production of these antibodies by mice was associated with aberrations in glucose homeostasis.[11] Two seemingly opposite effects were noted during fasting: hypoglycemia followed by hyperglycemia. The hypoglycemia, which appeared during the first week of anti-DM Id production, was explainable by stimulation of the insulin receptor by the antibody. The hypoglycemia was abruptly replaced by fasting hyperglycemia which persisted for 1–2 weeks until the anti-DM Id disappeared. The switch to hyperglycemia was associated with down-regulation (loss of about 50% or more of the insulin receptors on fat cells), profound desensitization (10-fold more insulin needed for half-maximal stimulation of lipogenesis by insulin), and refractoriness (40–80% reduction in maximal stimulation of lipogenesis by insulin).[5] We have recently discovered that it is possible to repair insulin receptor desensitization and refractoriness, but not down-regulation, by treating the mice with β_1-adrenergic agonists.[12] As expected, preserving the sensitivity of the insulin receptor led to persistent hypoglycemia caused by the insulinlike action of the anti-DM Id produced by the mice.

In addition to providing an *in vivo* model for studying regulation of receptor desensitization, this finding illustrates that a single species of antibody, the anti-DM Id, can produce either hyperglycemia or hypoglycemia depending on the degree of adaptive desensitization manifested by the insulin receptor. Indeed, anti-DM Id antibodies have been associated with intractable hypoglycemia in humans.[13] Antibodies to the insulin receptor also have been detected in patients with Type I diabetes mellitus,[14] and it seems that these antibodies may be anti-DM Ids (in preparation).

Immunological regulation of the DM Id–anti-DM Id network is another subject suitable for investigation provided by our observation. Why are anti-DM Ids the only spontaneous anti-idiotypic antibodies detectable after immunization of mice to insulin? Why is the DM Id confined to the primary response? Why do guinea pigs not make the anti-DM Id if they make the DM Id? [Perhaps the DM Id is not an autoantibody in guinea pigs, which have a markedly divergent structure of their insulin[11].] Why is

[11] I. R. Cohen, D. Elias, R. Maron, and Y. Shechter, *in* "Idiotypy in Biology and Medicine" (H. Köhler, J. Urbain, and P.-A. Cazenave, eds.), p. 385. Academic Press, New York, 1984.

[12] D. Elias, M. Rapoport, I. R. Cohen, and Y. Shechter, *J. Clin. Invest.* **81,** 1979 (1988).

[13] D. Elias, I. R. Cohen, Y. Shechter, Z. Spirer, and A. Golander, *Diabetes* **36,** 348 (1987).

[14] R. Maron, D. Elias, B. M. de Jongh, G. J. Bruining, J. J. van Rood, Y. Shechter, and I. R. Cohen, *Nature (London)* **303,** 817 (1983).

the DM Id shared by mice, guinea pigs, and humans? Does the anti-DM Id have a role in Type I or in Type II diabetes mellitus of humans? These and other questions can be answered with the aid of the monoclonal antibodies described here. The DM Id is a handy reagent for detecting anti-DM Id and, vice versa, the anti-DM Id detects the DM Id.

[21] Anti-idiotypic Antibodies as Probes of Prolactin Receptor

By MOUSSA B. H. YOUDIM, RONNIE J. BARKEY, T. AMIT, and J. GUY

Introduction

A principal action of prolactin (PRL) that is widely recognized and accepted is the stimulation of milk formation in the hormonally prepared female breast. However, it has been demonstrated that this hormone has an extremely wide spectrum of physiological effects in many species. Greater understanding of the neurotransmitter-mediated mechanism of PRL secretion and its central and peripheral endocrine action has made the hormone eminent and a subject of numerous studies.[1-3] Of interest to us is the up-regulation of PRL receptor by the hormone itself, a finding originally described by Posner *et al.*[4] and Djiane and Durand[5] in rabbit mammary gland and rat liver, respectively. This action is contrary to the inhibitory effect (down-regulation) of a large number of hormones on the level of their own receptor and suggests a special functional regulatory action of PRL in target tissues.

Surfactant synthesis in lung type II cells is increased by a number of hormones including glucocorticoids, thyroid hormone, and estrogen[6] in the last phases of gestation. It is at this time that circulatory PRL in-

[1] R. M. Macleod and U. Scapagnini, eds., "Central and Peripheral Regulation of Prolactin Function." Raven Press, New York, 1980.

[2] W. F. Ganong, *in* "Central and Peripheral Regulation of Prolactin Function" (R. M. Macleod and U. Scapagnini, eds.), p. 1. Raven Press, New York, 1980.

[3] C. S. Nicoll, B. A. White, and F. C. Leung, *in* "Central and Peripheral Regulation of Prolactin Function" (R. M. Macleod and U. S. Scapagnini, eds.), p. 11. Raven Press, New York, 1980.

[4] B. I. Posner, P. A. Kelly, and H. G. Friesen, *Science* **187,** 57 (1975).

[5] J. Djiane and P. Durand, *Nature (London)* **266,** 641 (1977).

[6] P. L. Ballard, *in* "Metabolic Activities of the Lung" (R. Porter and J. Whelan, eds.), p. 251. Elsevier, Amsterdam, 1980.

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