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Production of Insulinomimetic Antibodies Against Rat Adipocyte Membranes by Hybridoma Cells

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SJL mice were injected intraperitoneally with adipocyte plasma membranes or with intrinsic membrane proteins obtained by extraction of plasma membranes with dimethylmaleic anhydride. Three days after the boost injection, the spleens were removed and fused with NS-1, a thioguanine-resistant myeloma cell line derived from P_3X63 Ag8 (Balb/c). Following selection for hybrids with hypoxanthine, aminopterin, and thymidine, medium of the hybrid cells was tested for its ability to bind to the plasma membrane of the adipocyte and to stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$. Approximately 40% of the wells containing hybridomas derived from splenocytes of SJL mice immunized with plasma membranes produced immunoglobulin that bound to adipocyte plasma membranes. About 30% of these mimicked the ability of insulin to stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ in adipocytes. Media from 51% of the wells containing hybridomas derived from splenocytes of SJL mice immunized with intrinsic membrane proteins produced immunoglobulin that bound to the plasma membrane and 48% of those stimulated glucose oxidation. The bioactivity of the hybrid cell media could be blocked by adsorption with intrinsic membrane proteins or by the removal of immunoglobulins using formalin-fixed Staphylococcus aureus. The hybrids generated in this study can be divided into three categories: 1) hybrids that secrete antibodies that can bind to plasma membranes and mimic insulin action of glucose transport; 2) hybrids that secrete antibodies that bind to plasma membranes but do not stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$; and 3) hybrids that produce no antimembrane antibodies. The data suggest that interaction of immunoglobulins with specific membrane proteins is essential in mimicking the action of insulin on glucose transport and oxidation in the rat adipocyte.

Key words: hybridoma cells, insulin action, insulinomimetic antibodies

Insulin plays a central role in the regulation of cellular carbohydrate and fat metabolism. A number of agents including sulfhydryl reagents [1], lectins [2], trypsin [3], neuraminidase [4, 5], and antireceptor or antimembrane antibodies [6–10] have been shown to mimic the action of insulin on glucose transport and on conversion of D-(1-^{14}C) glucose to $^{14}CO_2$ in rat adipocytes. Kahn and co-workers [6] have shown that patients suffering from type B syndrome insulin resistance and acanthosis nigricans have antibodies in their sera that block insulin binding and mimic the action of insulin on stimulation of glu-

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cose uptake into rat adipocytes. Monovalent fragments of the immunoglobulin fraction from these sera are ineffective in stimulating glucose transport [7]. Antibodies prepared against a highly purified preparation of rat liver insulin receptor have been shown to immunoprecipitate the insulin receptor from solubilized rat liver membranes [8, 9] and to increase glucose uptake in 3T3-L1 cells [10]. Antibodies specific for rat adipocyte intrinsic membrane proteins can also mimic the action of insulin on glucose transport [11], whereas their monovalent Fab fragments prepared by papain digestion are ineffective in enhancing glucose uptake [12]. Biological activity of the Fab fragments could be restored by the addition of goat antirabbit Fab antisera to adipocytes treated with the Fab fraction of rabbit antimembrane IgG [12]. Antiintrinsic membrane protein antibodies can readily activate hexose uptake in trypsinized cells which have lost their capability to respond to insulin [11] and do not directly react with the insulin receptor or the glucose transporter [13]. These immunoglobulins do interact with an M_r = 94,000 adipocyte membrane glycoprotein which as yet has no known function.

Aggregation of membrane components by antireceptor antibodies, antiintrinsic membrane protein antibodies, or concanavalin A [2] has been suggested to be necessary for activation of the glucose transporter. However, in the cases of the latter two reagents it is not known whether general membrane perturbation of any intrinsic membrane protein is sufficient to mimic the action of insulin or if the interaction of specific proteins with antibody is required for stimulation. Monoclonal antibodies to the adipocyte plasma membrane should aid in evaluating this question, since these immunoglobulins are specific for one antigenic determinant. Many laboratories have generated monoclonal antibody-producing cells with specificity for cell surface antigens such as the acetylcholine receptor [14], Forsmann antigen [15], and HLA determinants [16]. In this report we describe the generation of hybridomas that produce antibodies that bind to the rat adipocyte plasma membrane and exhibit the ability to mimic the action of insulin on intact adipocytes by stimulating glucose oxidation.

METHODS

Preparation of Antigen

Fat cells were isolated from adult female or male rats as previously described [17] by collagenase digestion of parametrial fat pads. Isolated fat cells were washed with Krebs-Ringer phosphate buffer containing 2% bovine serum albumin and then homogenized in 0.25 M sucrose in 5 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. A crude plasma membrane fraction was collected by differential centrifugation and treated with dimethylmaleic anhydride, as previously published [18], to remove extrinsic membrane proteins from the membrane.

Immunization and Fusion

Female SJL mice were immunized by intraperitoneal injection of 50–100 μg rat adipocyte crude plasma membranes (1 mg/ml) or intrinsic membrane proteins (1 mg/ml) in Krebs-Ringer phosphate buffer. Booster injections of 50 μg membrane protein were given intraperitoneally 5 weeks later. Three days after the boost injections, spleens from mice immunized with membrane proteins were removed. Splenocytes were dissociated mechanically, washed and fused with NS-1, a mouse myeloma cell line derived from $P_3X63Ag8$, according to the procedure of Kohler and Milstein [19]. Splenocytes from mice immunized

with intrinsic membrane proteins and plasma membranes were used to generate hybridomas specific for intrinsic membrane and total membrane proteins, respectively. Spleen cells (2×10^8) were fused with 2×10^8 myeloma cells by the addition of 1 ml 50% polyethylene glycol 1500. After fusion, the cells were suspended in RPMI 1640 media containing 100 μm hypoxanthine, 1 μm aminopterin, 16 μm thymidine and 13% horse serum [20] and inoculated into wells at 10^6 cells/well. Half replacements of the medium were performed every 3 days. At 3–4 weeks, the cell media were assayed for presence of antiadipocyte plasma membrane antibodies.

Glucose Oxidation in Adipocytes

Production of $^{14}CO_2$ from D-(1-^{14}C) glucose was determined according to the procedure of Fain et al [21]. Packed fat cells suspended in Krebs-Ringer phosphate buffer containing 4% bovine serum albumin were added to plastic test tubes containing 50 μl of sera or hybridoma medium and 0.1 ml Krebs-Ringer phosphate buffer containing 4% bovine serum albumin and D-(1-^{14}C) glucose (New England Nuclear) (0.19–0.48 mM, 0.1–0.2 μCi/ml). After a 60-minute incubation at 37°C, the reaction was stopped by the addition of 0.2 ml of 1 N H_2SO_4 to the fat cells, and phenethylamine (0.2 ml) was added to pieces of Whatman filter paper suspended in plastic wells above the fat cells. The liberated $^{14}CO_2$, precipitated on the filter papers, was measured in a Beckman liquid scintillation counter using a solution of Triton X-100 in toluene (33% v/v) containing 4 g/liter Omnifluor (New England Nuclear). Controls containing RPMI 1640 medium, 1–6 milliunits insulin, rabbit antiintrinsic membrane proteins no RPMI 1640 medium, and no fat cells were also run.

The effect of removing immunoglobulins from the hybridoma media on glucose oxidation was also assayed. Cell medium (100 μl) was incubated with 2 μl rabbit antimouse immunoglobulin IgG (Cappel Laboratories) and 20 μl formalin-fixed Staphylococcus aureus (The Enzyme Center) for 1 hour at room temperature and centrifuged. The supernatants were removed and assayed for their ability to stimulate oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ as described above.

Medium of hybridomas generated from splenocytes immunized with plasma membranes was adsorbed with total plasma membrane proteins and intrinsic membrane proteins and then assayed for stimulation of glucose oxidation. Hybridoma medium (300 μl) was incubated with either 50 μl plasma membrane (11.2 mg/ml) or 30 μl intrinsic membrane proteins (3.2 mg/ml) overnight at 4°C. The mixture was centrifuged to pellet the membranes and adsorbed immunoglobulins and the supernatants were removed and assayed as described above for stimulation of glucose oxidation and for binding to plasma membrane.

Binding of Antibodies to Rat Adipocyte Plasma Membrane

Synthesis of antirat adipocyte membrane antibodies by hybridomas was measured by direct binding of antibodies to plasma membranes. Hybridoma cell medium or serum (50 μl) was incubated with 25 μl plasma membrane (1 mg/ml) overnight at 4°C, centrifuged at 3,000g for 10 minutes, and incubated for 2 hours at room temperature with 200 μl rabbit antimouse immunoglobulin IgG (Cappel Laboratories) at 2.6 mg/ml in Krebs-Ringer phosphate buffer containing 0.5% bovine serum albumin and centrifuged at 3,000 rpm for 10 minutes. The pellet was incubated for 30 minutes at room temperature with ^{125}I-protein A (1850 cpm/ng) (Pharmacia) that was iodinated with Enzymobeads (Bio-Rad), centrifuged at 3,000g for 10 minutes, washed, and the pellet was counted. Controls containing supplemented RPMI 1640 medium and rabbit antiintrinsic membrane protein antisera [11, 12] were also performed.

RESULTS

SJL mice injected and boosted with 50 μg rat adipocyte membrane proteins were bled retroorbitally 7 days after the boost and the serum was assayed for its ability to bind to rat adipocyte plasma membrane. As shown in Table I, serum obtained from mice injected with intrinsic membrane proteins or plasma membranes bound to adipocyte plasma membranes. Rabbit antiintrinsic membrane protein antisera previously shown to mimic insulin action [11] was used as a control to demonstrate high levels of ^{125}I-protein A binding. The values in Table I represent specific ^{125}I-protein A binding from which the nonspecific binding of ^{125}I-protein A to plasma membranes in the absence of antimembrane or control serum has been subtracted. The serum was also tested on intact adipocytes for its ability to activate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$. The serum was incubated at 56°C for 30 minutes to destroy complement activity prior to assay. Rabbit antiintrinsic membrane protein antisera (50 μl) and 2.4 milliunits/ml insulin maximally stimulated D-(1-^{14}C) glucose conversion to $^{14}CO_2$. As shown in Table I, mouse antiplasma membrane and antiintrinsic membrane protein antisera significantly stimulated the oxidation of D-(1-^{14}C) glucose conversion to $^{14}CO_2$ over that seen with control serum although not to the level seen in the presence of insulin or rabbit antimembrane antisera.

Spleen cells from SJL mice immunized with either plasma membrane or intrinsic membrane protein and NS-1 cells which lack hypoxanthine phosphoribosyl transferase activity (EC2.4.2.8) were fused in the presence of polyethylene glycol. Following selection for hybrids, colonies were present in 95% of the wells. Approximately 40% of the wells containing hybrids derived from splenocytes of SJL mice immunized with crude plasma membranes produced immunoglobulins that bound to ^{125}I-protein A and plasma membrane and 30% of these stimulated glucose oxidation.

Table II shows a typical distribution of values for binding of media to plasma membrane and stimulation of glucose oxidation. Medium that stimulated D-(1-^{14}C) glucose conversion to $^{14}CO_2$ to less than 15% of maximal level of oxidation obtained with addition of 1.2 milliunits of insulin was classified as medium that did not secrete bioactive antibody. Medium that exhibited greater than 10% specific binding of ^{125}I-protein A to plasma membrane after incubation with hybridoma medium and rabbit antimouse immunoglobulin IgG was considered as a positive binder. Thus, hybrids could be typed into those that secreted medium that 1) neither stimulated glucose oxidation nor bound to plasma membrane; 2) bound to plasma membrane but did not stimulate the conversion of D-(1-^{14}C) glucose to $^{14}CO_2$; and 3) both bound to plasma membrane and stimulated the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$.

These hybridomas were generated from splenocytes immunized with crude plasma membranes; thus, the immunoglobulins generated would be against both extrinsic and intrinsic membrane proteins. Hybridoma medium that stimulated glucose oxidation was adsorbed with either intrinsic membrane proteins or crude plasma membranes and then assayed for its ability to stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$. As can be seen in Table III, PMI-5H medium only minimally stimulates the oxidation of glucose but does bind to plasma membrane. After adsorption with either intrinsic or total membrane proteins, the medium stimulates glucose oxidation to the same extent as before adsorption. The values for glucose oxidation in Table III are corrected for basal oxidation in the absence of medium. The low level of stimulation seen in PMI-5H is also observed with supplemented RPMI 1640 medium which has not been exposed to cells. However, the stimulation is not due to immunoglobulins, as dialyzed medium does not stimulate glucose oxidation above basal levels (data not shown). PMI-9B medium contained immunoglobulin

TABLE I. Serum Activity of SJL Mice Immunized With Rat Adipocyte Intrinsic and Plasma Membrane Proteins

Additions	D-(1-^{14}C) glucose conversion to $^{14}CO_2$		Specific ^{125}I-protein A bound to plasma membrane
	Expt 1	Expt 2	
	nmoles/10^5 cells/60 minutes		(%)
None	0.9	0.6	–
Insulin, 2.4 milliunits/ml	13.5	11.1	–
Rabbit antimembrane serum[a], 50 μl	–	11.0	40
Control serum, 50 μl	–	0.6	5–14
Mouse antiintrinsic membrane antiserum			
50 μl	2.0	1.7	24–30
100 μl	3.6	–	–
Mouse antiplasma membrane antiserum			
50 μl	1.1	–	28
100 μl	1.6	–	–

[a]See reference [11].

Serum was obtained from SJL mice injected and boosted with 50 μg intrinsic membrane proteins or crude plasma membranes. Control serum was obtained from SJL mice immunized with saline. The serum was heated at 56°C for 30 minutes to destroy complement. Fat cells (75 μl, ~9 × 10^6 cells/ml) were incubated at 37°C for 1 hour with labeled and unlabeled D-(1-^{14}C) glucose (0.19 mM) and 50–100 μl serum in a final volume of 0.5 ml. Insulin (2.4 milliunits/ml) was added where indicated at the start of the incubation. Experiments 1 and 2 were conducted on different days with different serum. In order to monitor immunoglobulin binding, plasma membranes (25 μl, 1 mg/ml) were incubated with 50 μl sera overnight at 4°C and centrifuged. The pellet was incubated with rabbit antimouse immunoglobulin IgG, centrifuged, and incubated with ^{125}I-protein A (850 cpm/ng). The pellet contained ^{125}I-protein A bound to the plasma membrane via immunoglobulins. The values are corrected for nonspecific binding of ^{125}I-protein A to plasma membranes in the absence of serum.

that bound to the plasma membrane and stimulated the oxidation of glucose approximately 70% of maximal stimulation obtained in the presence of insulin and medium. Upon adsorption with intrinsic membrane proteins, both the binding to plasma membrane and the degree of oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ were decreased to control medium levels. Further adsorption of the medium with plasma membrane did not significantly decrease the level of glucose oxidation. The conclusion from this experiment is that the membrane components to which biologically active antibodies bind are present in the intrinsic membrane preparation.

Colonies were present in 261 out of 275 wells containing hybrids generated from splenocytes of mice immunized with intrinsic membrane proteins. Media harvested from 142 wells (51.6%) contained antibodies that bound to plasma membrane and rabbit antimouse immunoglobulin IgG. Medium that exhibited greater than 10% specific binding of ^{125}I-protein A to plasma membrane in the presence of rabbit antimouse immunoglobulin IgG was considered positive for binding to plasma membrane. Sixty-eight wells contained antibodies that stimulated the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ in intact adipocytes. Hybrids that secreted medium that stimulated glucose oxidation greater than 15% of maximal stimulation obtained with insulin were classified as exhibiting biological activity. A

TABLE II. Screening of Medium From Hybridomas Generated From Splenocytes of Plasma-Membrane-Immunized SJL Mice

Well	Type (see text)	Increment of D-(1-^{14}C) glucose converted to $^{14}CO_2$ over basal levels (nmoles/10^5 cells/60 minutes)	Specific ^{125}I-protein A bound to plasma membrane (%)
1-3E	I	2.5	0
1-4G	I	1.7	2.3
1-9D	I	4.1	3.5
1-10A	I	0	0
1-10D	I	0	4.4
1-7G	II	0	11.5
1-7H	II	0.5	25.5
1-8E	II	4.0	25.5
1-9A	II	0	10.0
1-10H	II	0	21.8
1-6H	III	33.3	20.0
1-7F	III	8.7	16.5
1-11G	III	14.8	20.2
Insulin, 2.4 mU/ml	–	34.2	ND[b]
Rabbit antimembrane serum[a]	–	ND[b]	39.8

[a]Reference [11].
[b]Not determined.
Fat cells (35 μl, ~9 × 10^6 cells/ml) were incubated at 37°C for 1 hour with labeled and unlabeled D-(1-^{14}C) glucose (0.48 mM) and 50 μl medium in a final volume of 0.2 ml. Insulin (6.0 milliunits/ml) was added where indicated at the start of the incubation. The values are corrected for oxidation due to supplemented RPMI 1640 medium. Binding of ^{125}I-protein A to plasma membranes via immunoglobulins was assayed as described in Table I using 50 μl hybridoma medium.

TABLE III. Adsorption of Antiplasma Membrane Antibodies With Intrinsic Membrane

Well	Adsorbed with	Increment of D-(1-^{14}C) glucose converted to $^{14}CO_2$ over basal levels (nmoles/10^5 cells/60 minutes)	Specific ^{125}I-protein A bound to plasma membrane (%)
PM1-5H	–	2.9	17
PM1-5H	Intrinsic membrane	2.5	15
PM1-5H	Plasma membrane	3.0	ND
PM1-5H	+ Insulin	8.6	ND
PM1-9B	–	5.5	6.6
PM1-9B	Intrinsic membrane	3.2	0.7
PM1-9B	Plasma membrane	2.8	ND
PM1-9B	+ Insulin	6.6	ND
Rabbit antimembrane antiserum[a]	–	ND	21

[a]Reference [11].
Hybridoma medium (300 μl) was incubated with either 50 μl plasma membranes (11.2 mg/ml) or 30 μl intrinsic membrane proteins (3.2 mg/ml) overnight at 4°C. The mixtures were centrifuged and the supernatants (50 μl) were assayed for stimulation of glucose oxidation and binding to plasma membranes as described in Table II. Glucose oxidation in control cells has been subtracted.

representative sample of the screening data is shown in Table IV. The media vary greatly in ability to bind to plasma membrane and to stimulate the oxidation of glucose, but can be divided into three categories: 1) media containing no binding or biological activity; 2) media containing antibody that binds plasma membranes but elicits no stimulation of glucose oxidation; and 3) media that contains antibodies that bind and stimulate the conversion of D-(1-^{14}C) glucose to $^{14}CO_2$. There does not appear to be a good correlation between the magnitude of immunoglobulin binding to plasma membrane and stimulation of glucose oxidation.

This ability of the medium to stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ was evaluated by testing its dependence upon the removal of immunoglobulins (Table V). Cell medium was incubated with rabbit antimouse immunoglobulin IgG and formalin-fixed Staphylococcus aureus for 1 hour at room temperature. The incubation mixture was centrifuged and the media was assayed for its ability to stimulate the oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$. The addition of rabbit antimouse immunoglobulin IgG is necessary for the detection of immunoglobulins of the IgM, IgA, and IgG_1 classes and facilitates the detection of the other subclasses of IgG [22]. The cell wall of Staphylococcus aureus con-

TABLE IV. Screening of Media From Hybridomas Generated From Spleens of SJL Mice Immunized With Intrinsic Membrane Proteins

Well	Type (see text)	Increment of D-(1-^{14}C) glucose conversion to $^{14}CO_2$ over basal levels (nmoles/10^5 cells/hour)	Specific ^{125}I-protein A bound to plasma membrane (%)
1-2F	I	1.6	4
1-2H	I	1.3	0
1-4D	I	1.5	3
1-5D	I	0	5
1-12A	I	1.1	4
1-12E	I	0.8	8
1-2B	II	1.6	28
1-2C	II	1.9	23
1-2D	II	0.8	24
1-2E	II	1.4	16
1-3B	II	1.5	23
1-4B	II	2.3	17
1-4C	II	2.9	22
1-12C	II	0.4	22
1-2G	III	4.8	18
1-3A	III	4.4	29
1-3C	III	6.2	19
1-3E	III	3.8	21
1-3F	III	6.7	31
1-5A	III	4.9	4[b]
1-5B	III	6.5	16
1-5G	III	4.3	20
Rabbit antimembrane antisera[a]	–	7.7	40
Insulin, 6 mU/ml	–	8.2	–

[a]Reference [11].

[b]Expressed binding to plasma membrane at a later date.

Hybridoma medium (50 μl) was assayed for stimulation of glucose oxidation and binding to plasma membrane as described in Table II.

TABLE V. Specific Removal of Immunoglobulins From Hybridoma Media Abolishes Biological Activity

Well	Increment of D-(1-^{14}C) glucose converted to $^{14}CO_2$ over basal levels	
	No treatment	Staph aureus-treated
	(nmoles/10^5 cells/60 minutes)	
1-5A	5.9	1.6
1-5B	7.3	1.1
1-5C	1.9	1.5
1-5D	1.4	1.0
1-5E	1.6	1.1
1-5G	5.5	1.7
1-5H	5.1	1.4
PMI-5H	2.9	2.7
PMI-9B	5.5	3.2
Control medium	1.1	1.3
2.4 mU/ml Insulin	8.7	–

Hybridoma medium (100 μl) was incubated with rabbit antimouse immunoglobulin IgG and formalin-fixed Staph aureus for 1 hour at room temperature and centrifuged. The supernatants were removed and tested for their ability to stimulate oxidation of D-(1-^{14}C) glucose to $^{14}CO_2$ in rat adipocytes as described in Table II. Glucose oxidation values are corrected for basal oxidation.

tains protein A, which binds specifically and strongly to the Fc regions of most subclasses of mammalian IgG. As can be seen in Table V, medium from wells 1-5A, 1-5B, 1-5G, 1-5H, and PMI-5H stimulated, respectively, the oxidation of glucose in adipocytes to 67%, 83%, 63%, 58%, and 63% of the maximal response observed with insulin. Removal of the immunoglobulin by Staphylococcus aureus precipitation decreased the level of glucose oxidation to background. This phenomenon is exhibited by media from hybrids generated against both intrinsic membrane proteins (1-5A, 1-5B, 1-5G, and 1-5H) and against plasma membranes (PMI-5H).

DISCUSSION

It has been previously reported that antisera generated in rabbits against rat intrinsic membrane proteins react with both mouse and rat adipocytes [12]. An immunoprecipitation reaction between these antisera and Triton X-100 solubilized mouse or rat adipocyte plasma membranes indicated a reaction of identity in immunodiffusion plates. Also, rabbit antiserum against rat adipocyte intrinsic membrane proteins caused stimulation of glucose oxidation in both mouse and rat adipocytes [12]. Insulin action on adipocyte lipolysis was also mimicked by the antibodies [12]. In the present studies, antiserum from SJL mice injected with membrane proteins was found to contain antibodies that bind to the plasma membrane and mimic the action of insulin as shown in Table I. While the stimulation of glucose oxidation is not so potent as previously observed in rabbit antisera, it is reproducible and dose-dependent (Table I). Mouse antiintrinsic membrane antiserum bound to adipocyte plasma membranes adsorbed ^{125}I-protein A to the same extent as mouse antiplasma membrane antisera. However, the former antisera stimulated glucose oxidation at least twofold better.

The media of many hybridomas resulting from the fusion of NS-1 cells with splenocytes immunized with intrinsic membrane proteins and plasma membranes were capable of stimulating glucose oxidation as shown in Tables IV and II, respectively. In most cases, this stimulating ability corresponded to the ability to bind rabbit antimouse immunoglobulin IgG. However, the values for binding to protein A and stimulating glucose oxidation do not directly correlate in magnitude. Protein A binds specifically to the Fc region of most mammalian IgG subclasses [22], but does not bind to mouse immunoglobulins with μ, α, or γ_1 heavy chains. The presence of rabbit antimouse immunoglobulin IgG is necessary to evaluate the presence of these immunoglobulins. Thus, the amount of ^{125}I-protein A bound to the plasma membrane via immunoglobulins is dependent not only on concentration of membrane antigen and antibody in medium, but also on class of immunoglobulin and titer of rabbit antisera to that class of mouse immunoglobulin.

Specific removal of immunoglobulins from the hybridoma medium resulted in a loss of biological activity as shown in Table V. Incubation of the medium with rabbit antimouse immunoglobulin IgG and Staphylococcus aureus, which is a protein A-bearing organism, removes immunoglobulin from the media. Protein A has a higher total affinity for IgG-antigen complexes than free IgG alone [22], owing to a faster association and much slower dissociation rates for the complex. Precipitation of lymphocyte lysates with Staphylococcus aureus and subsequent analysis of the precipitate by gel electrophoresis have established that only immunoglobulin is precipitated from lymphocytes by this technique [22]. Therefore, the specific adsorption of insulinomimetic activity by Staphylococcus aureus strongly suggests that the biological effect is mediated by immunoglobulins and not by another agent secreted by the cell, such as protease or an oxidizing compound.

The adsorption studies shown in Table III and discussed in reference [12] indicate that biologically active antibodies can be adsorbed from either hybridoma media or antisera, respectively, by adipocyte-intrinsic membrane proteins. The hybridoma medium that did not mimic the action of insulin on glucose oxidation retained its ability to bind to plasma membrane after adsorption with intrinsic membrane proteins, whereas that medium that stimulated glucose oxidation lost its ability to stimulate the adipocyte after adsorption with intrinsic membrane proteins. This result is a clear indication that antibodies that are insulinomimetic bind to components present in intrinsic membrane preparations. Thus, the insulinomimetic agents produced by the hybridoma cells are immunoglobulins with specificity for the intrinsic membrane proteins of the rat adipocyte. However, approximately one-half of the hybridomas that produce immunoglobulins against intrinsic membrane proteins are not capable of generating medium that stimulates glucose oxidation (Table IV). This could be due to insufficient antigen on the plasma membrane accessible to immunoglobulin or to an interaction with membrane antigens that cannot trigger an insulinomimetic response.

The nature of the antigenic sites bound by these hybridoma antibodies remains to be determined, but preliminary experiments indicate that the antigenic portion of the intrinsic membrane proteins may not be in the carbohydrate region of the major intrinsic membrane glycoprotein as in the case of the rabbit antiintrinsic membrane protein antisera [11]. However, the binding of these immunoglobulins to intrinsic membrane proteins exposed to the exofacial surface of the fat cell is not sufficient to increase glucose transport and subsequent oxidation to CO_2 (Tables II and IV). This would indicate that specific protein interactions are necessary for activation of the glucose transporter. The exact location and extent of binding of the monoclonal immunoglobulins to the intact adipocyte is under investigation.

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