PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/575, 16/26, C12N 15/86, A61K 38/22

A2

(11) International Publication Number: **WO 98/24896**

(43) International Publication Date: 11 June 1998 (11.06.98)

(21) International Application Number: PCT/EP97/06541

(22) International Filing Date: 22 November 1997 (22.11.97)

(30) Priority Data:
96119606.0 6 December 1996 (06.12.96) EP
(34) Countries for which the regional or international application was filed: CH et al.

(71) Applicant: F. HOFFMANN–LA ROCHE AG [CH/CH]; Grenzacherstrasse 124, CH–4070 Basle (CH).

(72) Inventors: DEVOS, René; Kaaistraat 24, B–8400 Oostende (BE). GUISEZ, Yves; Zandstraat 209, B–8200 St–Andries/Brugge (BE). PLAETINCK, Geert; Pontstraat 16, B–9820 Bottelare (BE). VAN DER HEYDEN, José; Zink 23, B–9820 Munte (BE). VERPLOEGEN, Sandra; Hoge Noordweg 6A, NL–2671 DX Naaldwijk (NL).

(74) Agent: WITTE, Hubert; Grenzacherstrasse 124, CH–4070 Basle (CH).

(81) Designated States: AU, BR, CA, CN, CZ, HU, ID, IL, JP, KR, LT, LV, MX, NO, NZ, PL, RO, RU, SG, SI, TR, YU, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MUTEINS OF OBESE PROTEIN

(57) Abstract

The present invention relates to ob muteins, to DNA sequences coding for these ob muteins and methods for making these.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

MUTEINS OF OBESE PROTEIN

Obese protein (leptin), a fat secreted hormone, plays a central role in the control of body adiposity. Genetic defects that lead to obese protein deficiency (ob/ob mice) or obese protein (ob) resistance (db/db mice, fa/fa rats) both cause severe obesity (Zhang et al., Nature 372, 425-431 [1994]; Lee et al., Nature 379, 623-635 [1996]; Chen et al., Cell 84, 546-549 [1996]). Administration of recombinant ob protein decreases food intake and increases energy expenditure in ob/ob mice and WT mice (Campfield et al., Science 269, 546-549 [1995]; Halaas et al., Science 269, 543-546 [1995]; Pelleymounter et al., Science 269, 540-543 [1995]). The weight reducing effects are likely mediated through a signaling leptin receptor in the hypothalamus (Tartaglia et al., Cell 83, 1263-1271 [1995]).

Ob muteins with in vivo antagonistic properties have now been discovered. Administration of such muteins results in progressive increase in body weight. The ob muteins are thus of therapeutic use for disorders accompanying chronic diseases and wasting disorders, such as anorexia and cachexia, where weight gain would be beneficial.

The present invention thus provides ob muteins with normal receptor binding activity in a competition assay and with the inability to transduce biological signals. Preferred ob muteins of the present invention are those comprising SEQ ID No: 1, wherein at position 127 Ser is Asp or at position 128 Arg is Gln, with the designations S127D and R128Q, respectively.

The ob muteins of the present invention may contain at position 127 or 128 amino acid residues other than Asp or Gln, respectively, if such substitutions do not generally alter their binding activity and inability to transduce biological signals. Amino acid residues at position 127 or 128 may be selected from the group of the α-amino acids commonly found in nature excluding amino acid residues Ser and Arg.

The ob muteins of the present invention can contain specific sequences that preferably bind to an affinity carrier material. Examples of such sequences are sequences containing at least two adjacent histidine residues (see in this respect European Patent No. 282 042). Such sequences bind selectively to nitrilotriacetic acid nickel chelate resins (Hochuli and Döbeli, Biol. Chem. Hoppe-Seyler 368, 748 [1987]; European Patent No. 253 303). Ob

muteins which contain such a specific sequence can, therefore, be separated selectively from the remaining polypeptides. The specific sequence can be linked either to the C-terminus or the N-terminus of the ob mutein amino acid sequences.

The ob muteins may also have one or more chemical moieties attached to their amino acid sequences, including water soluble polymers such as polyethylene glycol. Polyethylene glycol derivatized derivatives can be mono-, di-, tri- or tetrapegylated, e.g., N-terminal monopegylated. Preferred pegylated derivatives of ob muteins of the present invention include ob muteins comprising SEQ ID NO:1, wherein at position 127 Ser is Asp or at position 128 Arg is Gln.

The present invention also provides DNA sequences which code for the ob muteins of the present invention, expression vectors which contain these DNA sequences, host cells containing such vectors for the production of the ob muteins and processes for the production of such DNA sequences, recombinant vectors and host cells. Methods for the expression, isolation and purification of the ob muteins are also described.

Because the complete DNA sequence of the gene coding for the natural ob protein (hereinafter also referred to as human leptin, hL) is known (Zhang et al., supra) amino acid sequences coding for the ob muteins of the present invention can be chemically synthesized using standard methods known in the art, preferably solid state methods, such as the methods of Merrifield (J. Am. Chem. Soc. 85, 2149-2154 [1963]). Alternatively, the ob muteins of the present invention can be produced using methods of DNA recombinant technology (Maniatis et al. in "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory [1982]). Preferably, a DNA sequence coding for an ob mutein is prepared from DNA coding for the natural ob protein and subsequently incorporated into a suitable expression vector which produces the requisite expression signals.

Expression vectors suitable for use in prokaryotic host cells are mentioned, for example, in the aforementioned textbook of Maniatis et al. Especially suitable vectors are plasmids of the pDS family (Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc., Vol. 155, 416-433 [1987]).

Such prokaryotic expression vectors, which contain the DNA sequences coding for the ob muteins of the present invention operatively linked with an expression control sequence, can be incorporated using conventional methods into any suitable prokaryotic host cell. The selection of a suitable prokaryotic host cell is determined by different factors which are well-known in the art. Thus, for example, compatibility with the chosen vector, toxicity of the expression product, expression characteristics, necessary biological safety precautions and costs play a role and a compromise between all of these factors must be found.

Suitable prokaryotic host organisms include gram-negative and gram-positive bacteria, for example E.coli and B.subtilis strains. Useful E.coli strains are E.coli M15 (described as strain OZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 [1974] and E.coli W3110 [ATTC No. 27325]). In addition to the aforementioned E.coli strain, however, other generally accessible E.coli strains such as E.coli 294 (ATCC No. 31446) and E.coli RR1 (ATCC No. 31343) can also be used.

Expression vectors suitable for use in mammalian hosts cells include but are not limited to pBC12MI, pBC12BI, pSV2dhFr, p91023(B), pcDV1, pRSVcat, pGA291, pGA293, pGA296, pBC12/HIV/IL-2 and pGA300.

Such vectors are preferably introduced into suitable mammalian host cells by transfection.

Mammalian host cells that could be used include, e.g., human Hela, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, CV1 African green monkey kidney cells, quail QC1-3 cells, Chinese hamster ovary (CHO) cells, mouse L cells and the COS cell lines.

The manner in which the expression of the ob muteins of the present invention is carried out depends on the chosen expression vector/host cell system.

Usually, the prokaryotic host organisms which contain a desired expression vector are grown under conditions which are optimal for the growth of the prokaryotic host organisms. At the end of the exponential growth, when the increase in cell number per unit time decreases, the expression of the desired ob mutein is induced, i.e., the DNA coding for the desired ob mutein is transcribed and the transcribed mRNA is translated. The induction can be carried out by adding an inducer or a derepressor to

the growth medium or by altering a physical parameter, e.g., a change in temperature.

The mammalian host cells which contain a desired expression vector are grown under conditions which are optimal for the growth of the mammalian host cells. A typical expression vector contains the promoter element, which mediates the transcription of mRNA, the protein coding sequence, and the signals required for efficient termination and polyadenylation of the transcript. Additional elements may include enhancers and intervening sequences bounded by spliced donor and acceptor sites.

Most of the vectors used for the transient expression of a given coding sequence carry the SV40 origin of replication, which allows them to replicate to high copy numbers in cells (e.g. COS cells) that constitutively express the T antigen required to initiate viral DNA synthesis. Transient expression is not limited to COS cells. Any mammalian cell line that can be transfected can be utilized for this purpose. Elements that control a high efficient transcription include the early or the late promoters from SV40 and the long terminal repeats (LTRs) from retroviruses, e.g. RSV, HIV, HTLVI. However, also cellular signals can be used (e.g. human-β-actin promoter).

Alternatively stable cell lines carrying a gene of interest integrated into the chromosome can be selected upon co-transfection with a selectable marker such as gpt, dhfr, neomycin or hygromycin.

Now, the transfected gene can be amplified to express large quantities of a foreign protein. The dihydrofolate reductase (DHFR) is a useful marker to develop lines of cells carrying more than 1000 copies of the gene of interest. The mammalian cells are grown in increasing amounts of methotrexate. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome.

The baculovirus-insect cell vector system can also be used for the production of the ob muteins of the present invention (for review see Luclow and Summers, Bio/Technology 6, 47-55 [1988]). The ob muteins produced in insect cells infected with recombinant baculovirus can undergo post-translational processing including N-glycosylation (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408) and O-glycosylation (Thomsen et al., 12.

International Herpesvirus Workshop, University of Philadelphia, Pennsylvania).

For the isolation of small amounts of ob muteins expressed in prokaryotic host cells for analytical purposes, e.g. for polyacrylamide gel electrophoresis, the host cells can be disrupted by treatment with a detergent, e.g., sodium dodecyl sulphate (SDS). Larger quantities of these ob muteins can be obtained by mechanical (Charm et al., Meth. Enzymol. 22, 476-556]1971]), enzymatic (lysozyme treatment) or chemical (detergent treatment, urea or guanidinium hydrochloride treatment, etc.) treatments followed by use of know methods, e.g. by centrifugation at different gravities, precipitation with ammonium sulphate, dialysis (at normal pressure or at reduced pressure), preparative isoelectric focusing, preparative gel electrophoresis or by various chromatogrpahic methods such as gel filtration, high performance liquid chromatography (HPLC), ion exchange chromatogrpahy, reverse phase chromatography and affinity chromatography (e.g. on Sepharose® Blue CL-6B or on carrier-bound monoclonal antibodies which are directed against the native ob protein and the ob muteins of the present invention).

The ob muteins expressed in mammalian host cells or in the baculovirus-insect cell vector system can be isolated from the host cell medium using standard protein purification methods.

The ob muteins can be used for the preparations of pharmaceutical compositions and for the treatment of disorders accompanying chronic diseases, e.g., long-term anorexia, anorexia of infection, cachexia and wasting disorders, e.g., cancer and AIDS and may prove their applicability in patients with anorexia nervosa.

They may be administered in pharmaceutically acceptable oral, injectable or topical compositions and modes. Dosage and dose rate may parallel that currently being used in clinical applications of the natural ob proteins. The pharmaceutical compositions of the present invention contain ob muteins, optionally in association with a monoclonal antibody against an ob mutein of the present invention and a compatible pharmaceutically acceptable carrier material. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for enteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate,

talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additonal additives such as flavouring agents, preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

The pharmaceutical preparations can be made up in any conventional form including: a) a solid form for oral administration such as tablets, capsules, pills, powders, granules and the like; b) a liquid form for oral administration such as solutions, syrups, suspensions, elixirs and the like; c) preparations for parenteral administration such as sterile solutions, suspensions or emulsions; and d) preparations for topical administrations such as solutions, suspensions, ointments, creams, gels, micronized powders, aerosols and the like. The pharmaceutical preparations may be sterilized and/or may contain adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, salts for varying the osmotic pressure and/or buffers.

Parenteral dosage forms may be infusions or injectable solutions which can be injected intravenously or intramuscularly. These preparations can also contain other medicinally active substances. Additional additives such as preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

Furthermore, antibodies can be raised against the ob muteins of the present invention. These antibodies can be used in a well-known manner for diagnostic or therapeutic purposes, for purification purposes, and for potentiating biological effects of the ob muteins. Such antibodies can be produced by injecting a mammalian or avian animal with a sufficient amount of a vaccine formulation comprising an ob mutein of the present invention and a compatible pharmaceutical carrier to elicit the production of antibodies against said mutein. The appropriate amount of the ob mutein which would be required would be known to one of skill in the art or could be determined by routine experimentation. As used in connection with this invention, the term "pharmaceutical carrier" can mean either the standard compositions which are suitable for human administration or the typical adjuvants employed in animal vaccinations.

Suitable adjuvants for the vaccination of animals include but are not limited to Freund's complete or incomplete adjuvant (not suitable for human or livestock use). Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N_1-N-N-dioctadecyl-N'-N-bis(2-hydroxyethylpropanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid, carbopol; peptides such as muramyl dipeptide, dimentylglycine, tuftsin; oil emulsions; and TiterMax. The soluble ob muteins could also be administered following incorporation into liposomes or other microcarriers, or after conjugation to polysaccharides, other proteins or other polymers or in combination with Quil-A to form "Iscoms" (immunostimulating complexes) (Morein et al., Nature 308, 457 [1984]). The adjuvants TiterMax is preferred (Vaxcel Inc., 3000 Northwoods Parkway, Norcross, GA 30071, USA).

Typically, the initial vaccination is followed some weeks later by one or more "booster" vaccinations, the net effect of which is the production of high titers of antibodies against the ob muteins which can be harvested in the usual way.

Another method consists in using the well-known Koehler and Milstein technique for producing monoclonal antibodies. In order to find out different monoclonal antibodies which are directed against the same antigen but against different epitopes, the method of Stähli et al. (J. of Immunological Methods 32, 297-304 [1980]) can be used.

Finally, the ob muteins of the present invention are useful in screening methods for identifying ob protein agonists.

It has also been discovered that the use of leptin or the ob muteins of the present invention in combination with antibodies against leptin, preferably in combination with monoclonal antibodies against leptin, potentiates the effects of these proteins. Thus, the present invention provides in addition the use of the ob muteins of the present invention in association with antibodies against leptin, preferably monoclonal antibodies against leptin, for the preparation of pharmaceutical compositions and for the treatment of disorders accompanying chronic diseases, e.g., long-term anorexia and anorexia of infection cachexia, and wasting disorders, e.g. cancer and AIDS.

The pharmaceutical compositions containing the ob muteins of the present invention in association with antibodies against leptin may be formulated and utilized as described hereinbefore.

Additionally, the present invention provides the use of human leptin (human obese protein) in association with antibodies against leptin, preferably monoclonal antibodies against leptin, for the preparation of pharmaceutical compositions and for the treatment, prevention and control of obesity and associated diseases.

The pharmaceutical compositions containing the human leptin in association with antibodies against leptin may be formulated and utilized as described in European Patent Application, Publication No. 741 187, or as described hereinbefore.

The preparation of human leptin is known, for example, from European Patent Application, Publication No. 741 187 and UK Patent Application 2 292 382. The term "human leptin (human obese protein)" includes all types of human leptin described in these applications.

Antibodies against the human leptin (human obese protein) can be produced as described hereinbefore or as described in European Patent Application, Publication No. 741 187 and UK Patent Application 2 292 382.

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures:

Fig. 1 shows ^{125}I hL displacement by cold wild-type hL and ob muteins on COS-1 cells transiently transfected with pSVSport-mLRsh. Each point represents mean ± s.e.m. cpm bound ^{125}I-hL (n = 3).

Fig. 2 shows effects of wild-type hL and ob muteins in the transfected BA/F3 proliferation assay. The data express mean ± s.e.m. cpm incorporated ^{3}H thymidine.

Fig. 3 shows in vivo biological effects of S127D and R128Q in combination with 2A5 mAb in ob/ob mice. Data are expressed as mean weight change ± s.e.m. (n = 3).

Fig. 4 shows in vivo antagonistic effect of R128Q in combination with 2A5 mAb in C57BL/6 mice. Data are displayed in mean change of body weight per group ± s.e.m. (n = 3).

Fig. 5 shows in vivo antagonistic effect of R128Q in combination with 2A5 mAb in C57BL/6 mice. Data are displayed in mean change of body weight per group ± s.e.m. (n = 3).

Fig. 6 shows the effect of R128Q on the serum insulin levels in C57BL/6 mice.

Fig. 7 shows in vivo potentiating effect of hL in ob/ob mice (1 injection/ day; 10 µg hL / injection; 1 mg 2A5 mAb/ injection). Data are displayed in mean change of body weight per group ± s.e.m. (n = 3).

Fig. 8 shows the effect of 2A5 mAb on hL in C37/B16 mice. C57/B16 mice were injected intraperitonialy (2 mice/group) with 80 ng ^{125}I labeled hL/10 µg cold hL with or without 0.2 mg 2A5 mAb. After the indicated time points mice were sacrificed and total blood was taken. 7 µl of serum was analysed by 15% PAGE and autoradiography. The signals one the autoradiograph were quantified with a phospho-imager.

Example 1

Identification of ob muteins

In order to identify individual residues involved in receptor binding, 37 single mutations (among them 29 resulting in changing charged to uncharged residues) were generated in the human ob protein cDNA by an adapted protocol using a commercially available kit (Transformer, Clontech) and were introduced in plasmid pSVSport (BRL). After transfection in COS-1 cells the expression of the muteins in the cell supernatant was quantified with an ELISA, using combinations of mAbs and polyclonal rabbit anti-serum.

All muteins were tested in a competition assay by measuring the displacement of ^{125}I hL (prepared by the Iodogen method, Pierce Chemical Co.) from the membrane anchored mouse leptin receptor short form (mLRsh) (Tartaglia et al., Cell 83, 1263-1271 [1995]), expressed on the surface of COS-1 cells. 2x10^6 transfected COS-1 cells/ml were incubated with 1nM ^{125}I-hL together with variable concentrations (2.5 ng/ml - 5 µg/ml) of

unlabelled wild-type hL or ob muteins for 3-5 hours at 4°C. Bound ligand was separated from free radioactivity by centrifugation through a phthalate oil cushion and γ emission of the pellet was counted. All solutions were made in Dulbecco's modified Eagle's medium (GibcoBRL) supplemented with 10% FCS, glutamine and gentamycin.

The biological activity was evaluated in an in vitro assay based on the proliferatation of leptin dependent BA/F3 cells. BA/F3 cells were transfected with a construct encoding a chimeric membrane anchored receptor which had been constructed by fusing the extracellular and the transmembrane domains of the mouse leptin receptor with the intracellular part of the human β_c chain (c stands for common), a subunit which is indispensable for GM-CSF/IL-3/IL-5 signal transduction (Tavernier et al., Cell 66, 1175-1184 [1991]). After selection clones have been obtained which are leptin dependent for their growth. In order to lower background proliferation 21F3 can be added, a monoclonal which had been developed against the related β_c chain (referred to as AIC2B; this chain is constitutively present in BA/F3 cells). After overnight incubation at 37°C without growth factor (=leptin), the leptin dependent BA/F3 cells (200 µl of cells; $1x10^3$ cells/point) were incubated in 96-well microtiter plates with variable concentrations of wild-type hL or ob muteins (0.05 - 100 ng/ml). After 72 h 0.5 µCi ^3H thymidine was added for 4 hours. The cells were harvested and the incorporated label counted.

Most of the ob muteins exhibited normal receptor binding and had similar specific biological activities as the wild-type protein. However, four muteins, R20Q, D40N, S127D and R128Q showed aberrant features. Since further detailed investigation depended on the availability of more pure product, the four mutated genes as well as the wild-type hL gene were introduced in the pVL1393 vector and were integrated into the Autographa californica nuclear polyhedrosis virus genome by cotransfection with linearized baculovirus DNA (Baculogold, PharMingen) in Sf9 cells as described (Summers and Smith, G.E. A [1987], Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures (Texas A & M University)). The proteins were purified from the insect cell medium on a 2A5-mAb column. Purity was evaluated on Coomassie-stained SDS-PAA gels (Laemmli, Nature 227, 680-685 [1970]) while protein concentrations were measured according to Bradford, Anal. Biochem. 72, 248-254 [1976], using a BioRad kit with BSA as a standard. Leptin proteins and ob muteins were

assayed in the displacement test (Fig. 1) as well as in the BA/F3 proliferation assay (Fig. 2).

Screening allowed to identify one residue involved in receptor binding, since an arginine replacement at position 20 by a glutamine (R20Q) abolished any displacement of ^{125}I hL in the competition assay (Fig. 1). The other purified muteins D40N, S127D and R128Q were still able to displace the radioactive ligand and did not significantly differ from wild-type hL in this respect, suggesting normal receptor binding. However, when the muteins were checked in the modified BA/F3 assay and compared with wild-type hL, reduced biological activities was found (Fig 2). As expected, R20Q, the mutant deficient in receptor interaction, showed no biological effect; the proliferation activity of D40N was reduced to about 50%. Surprisingly almost no proliferative response of S127D was detected on transfected BA/F3 cells, while R128Q showed no biological activity at all, even at a concentration of 100 ng/ml.

The antagonistic properties of the ob muteins S127D and R128Q were further illustrated on ob protein dependent BA/F3 cells, since proliferative responses were totally suppressed in the presence of 10 ng/ml wild-type hL.

Example 2

In vivo biological effects of R127D and R128Q in ob/ob mice

In order to evaluate the in vivo effects of the muteins, S127D, resp. R128Q, wild-type hL and PBS were intraperitoneally (i.p.) injected into ob/ob mice together with mAb 2A5. 6-8 week old ob/ob mice were injected with 15 µg wild-type hL, respectively 15 µg S127D, 15 µg R128Q and PBS (control). Injections were given daily i.p. for nine days in addition of mAb 2A5 (1.8 mg/injection). Body weight was measured before the first dose and at the same time on each subsequent day. Treatment with wild-type hL protein led to significant weight loss (up to 1g/day), while no significant effect was seen in animals injected with S127D, R128Q and PBS (Fig. 3). On the contrary, a slight body weight gain could be observed in these cases. A plausible explanation for this phenomenon is growth of the eight weeks old mice during the course of the experiment. Taken together, the injections in ob/ob mice unambiguously demonstrate the biological inactivity of R128Q and S127D, a fact which is in full accordance with the lack to trigger in vitro proliferative responses on modified BA/F3 cells (Fig 2).

Example 3

In vivo biological effects of R128Q in wild-type mice

Nine 9-10 week old C57BL/6 mice were divided in three equal groups and respectively injected intraperitoneally twice-daily (9.30 a.m. and 5.30 p.m.) with wild-type hL (100 µg/injection), ob mutein R128Q (100 µg/injection) and PBS (control) in the presence of 1.38 mg antibody 2A5 per injection. Body mass was daily determined by weighing before the first injection at 9.30 a.m.

Animals gained about 20% of their initial body weight after 15 days (Fig. 4) and after treatment had been stopped, returned to their initial weight (Fig. 5). Immediately after the 15-day treatment, the animals from the first experiment (Fig. 4) were sacrified and dissected. Abdominal fat depositions were clearly increased. Blood samples were taken and the insulin concentrations determined according to Morgen (Diabetes 12, 115 [1963]), using an insulin RIA kit (Linco Research, St. Charles). As can be seen in Fig. 6, insulin levels were significantly elevated in the wild-type mice injected with ob mutein R128Q compared to the controls, while hL injections had an opposite effect. The effects seen with i.p. injected wild-type hL are in contrast with the data obtained by Schwartz et al., J. Clin. Invest. 98, 1101-1106 (1996) with rats, where intracerebroventricularly administered leptin apparently did not alter insulin levels.

From the foregoing observations it is concluded that ob muteins S127D and R128Q behave as leptin antagonists with strong in vivo dominant negative effects.

Example 4

Potentiating effects of monoclonal antibodies

Treatment of C57BL/6 mice, that are homozygous (ob/ob) for the obese gene mutation (Zhang et al., supra), with exogenous leptin decreases food intake, increases physical activity and causes weight-reducing effects (Halaas et al., supra; Pelleymounter et al., supra; Campfield et al., supra).

Co-injection of recombinant hL with mAb 2A5 had clearly potentiating biological effects in ob/ob mice, which means that less exogenous leptin is needed to obtain the same beneficial effects (weight-loss) when co-administered with the antibody (Fig. 7).

The observed phenomenon is due to a longer leptin half-life in the serum probably by stabilizing or protecting effects caused by the antibody (Fig. 8). Monoclonal antibody (mAb) 2A5 is a mouse IgG mAb which was raised against hL by the well-known Koehler and Milstein technique.

Weight-reducing effects in wild-type mice require relatively high doses of exogenous leptin (Halaas et al., Science 269, 543-546 [1995]), apparently not due to the lack of post-translational modifications of the recombinant protein but as a result of pharmacokinetics (Cohen et al., Nature 382, 589 [1996]). Again, in our experiments the biological effects of exogenously administered hL in C57BL/6 mice (without ob gene mutations) are clearly potentiated by co-injection with mAb 2A5. As can be seen from Fig. 4, the animals lost about 15% of their initial body weight upon a 15-day treatment and maintained this plateau as long as the injections with wild-type hL lasted. In a second experiment, the animals were treated for nine days and their body weight further recorded even after the injections had been stopped. From Fig. 5 it can be deduced that the animals returned to their initial weight. These observations are in accordance with the concept in which leptin functions as a circulating "virtual fat messenger" and with the "set point" hypothesis on the long-term regulation of energy balance and maintenance of body weight (Keesey, In Obesity, A. Stunkard, eds. (Philadelphia: W.B. Saunders Co.), 144-166 [1980]; Harris, R.B.S. FASEB J. 4, 3310-3318 [1990]).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: F. HOFFMANN-LA ROCHE AG

(B) STREET: Grenzacherstrasse 124

(C) CITY: Basle

(D) STATE: BS

(E) COUNTRY: Switzerland

(F) POSTAL CODE (ZIP): CH-4070

(G) TELEPHONE: 061 - 688 42 56

(H) TELEFAX: 061 - 688 13 95

(I) TELEX: 962292/965542 hlr ch

(ii) TITLE OF INVENTION: Obese muteins

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: System 7.1 (Macintosh)

(D) SOFTWARE: Word 5.0

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1 5 10 15

Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
20 25 30

Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Ile
35 40 45

Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile
50 55 60

Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu
65 70 75 80

Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys
85 90 95

His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly
100 105 110

Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
115 120 125

Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
130 135 140

Gly Cys
145

Claims

1. An ob mutein with normal receptor binding activity in a competition assay and with the inability to transduce biological signals.

2. An ob mutein of claim 1 comprising SEQ ID No: 1, wherein at position 127 Ser is Asp or at position 128 Arg is Gln.

3. An ob mutein of claim 1 which is protein R128Q.

4. A DNA sequence coding for an ob mutein as claimed in claims 1 to 3.

5. An expression vector comprising a DNA sequence as claimed in claim 4 and being capable of directing expression of the DNA sequence in a compatible prokaryotic or mammalian host cell.

6. A prokaryotic or mammalian host cell transformed with an expression vector of claim 5.

7. A recombinant baculovirus comprising a DNA sequence as claimed in claim 5.

8. An insect host cell infected with a recombinant baculovirus of claim 7.

9. A method for the production of an ob mutein as claimed in claims 1 to 3 comprising culturing a transformed prokaryotic or mammalian host cell in a suitable medium so that said mutein is expressed and purifying said mutein.

10. A method for the production of an ob mutein as claimed in claims 1 to 3 comprising culturing an infected insect host cell in a suitable medium so that said mutein is expressed and purifying said mutein from the insect cell medium.

11. An ob mutein as claimed in claims 1-3 as a therapeutically active agent.

12. An ob mutein as claimed in claims 1-3 as a therapeutically active agent for the treatment of disorders accompanying chronic diseases and wasting disorders.

13. A pharmaceutical composition comprising an ob mutein as claimed in claims 1-3, optionally a monoclonal antibody against an obese protein and a compatible pharmaceutically acceptable carrier material.

14. The use of an ob mutein as claimed in claims 1-3 optionally in combination with a monoclonal antibody against an obese protein, for the preparation of pharmaceutical compositions.

15. The use of an ob mutein as claimed in claims 1-3, optionally in combination with a monoclonal antibody against an obese protein, for the preparation of pharmaceutical compositions for the treatment of disorders accompanying chronic diseases and wasting disorders.

16. Poly- and/or monoclonal antibodies raised against an ob mutein as claimed in claims 1-3.

17. An ob mutein as claimed in claims 1-3 whenever prepared by a process as claimed in claims 9 and 10.

18. A pharmaceutical composition comprising a human obese protein in association with a monoclonal antibody against said protein and a compatible pharmaceutically acceptable carrier material.

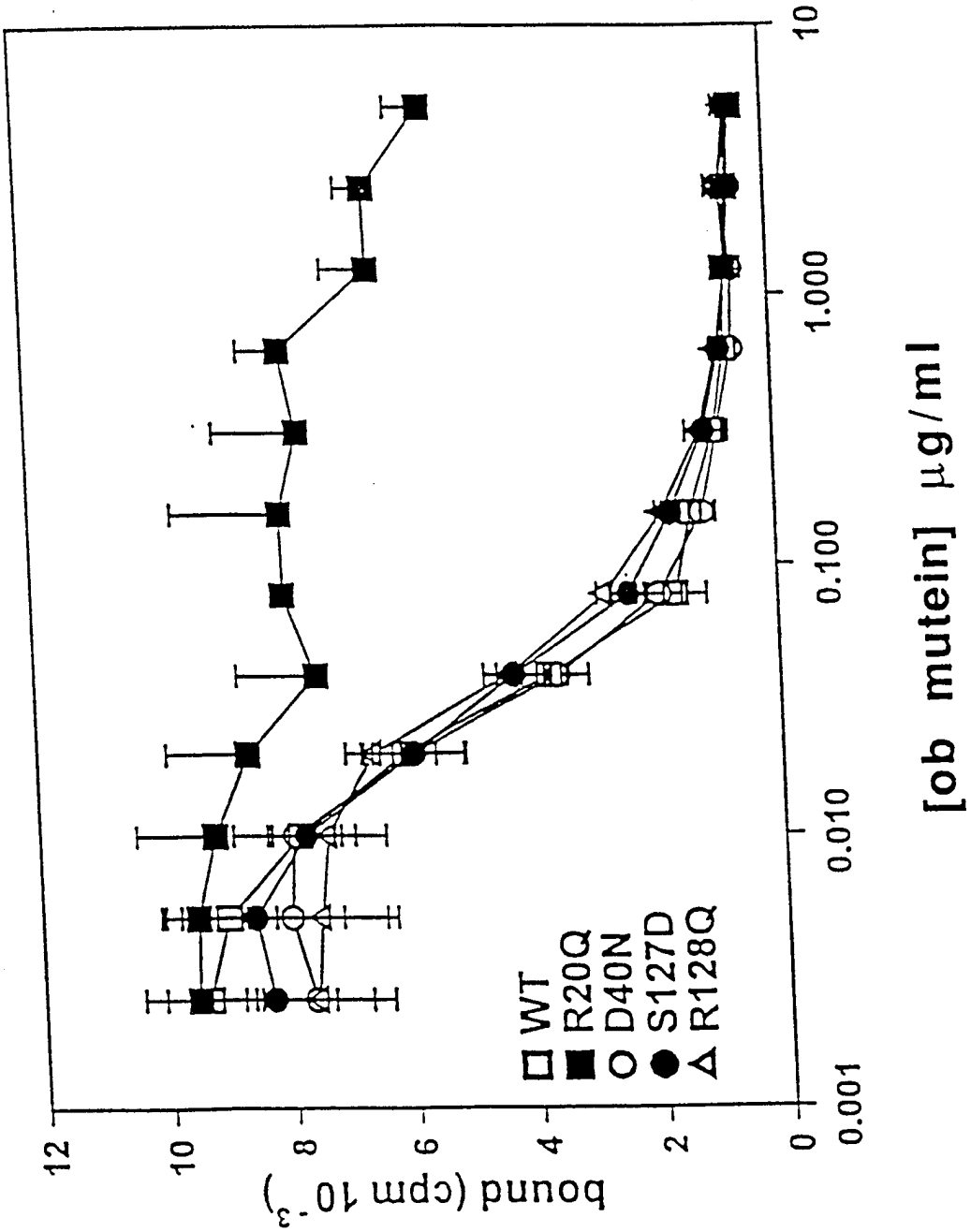
19. The use of a human obese protein in association with a monoclonal antibody against said protein for the preparation of pharmaceutical compositions.

20. The use of a human obese protein in association with a monoclonal antibody against said protein for the preparation of pharmaceutical compositions for the treatment, prevention and control of obesity and associated diseases.

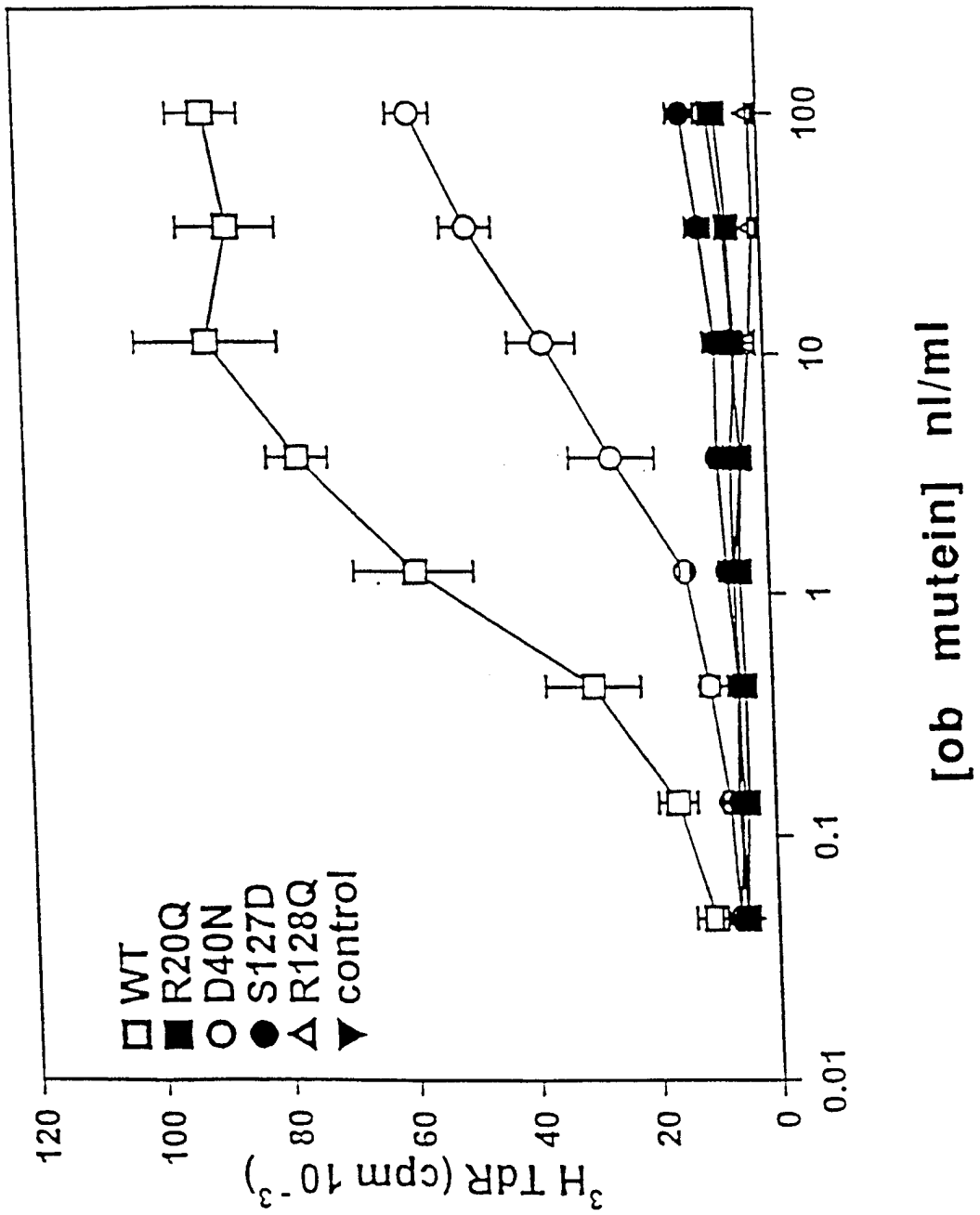
21. The products, pharmaceutical compositions, processes and methods substantially as described hereinbefore.

***

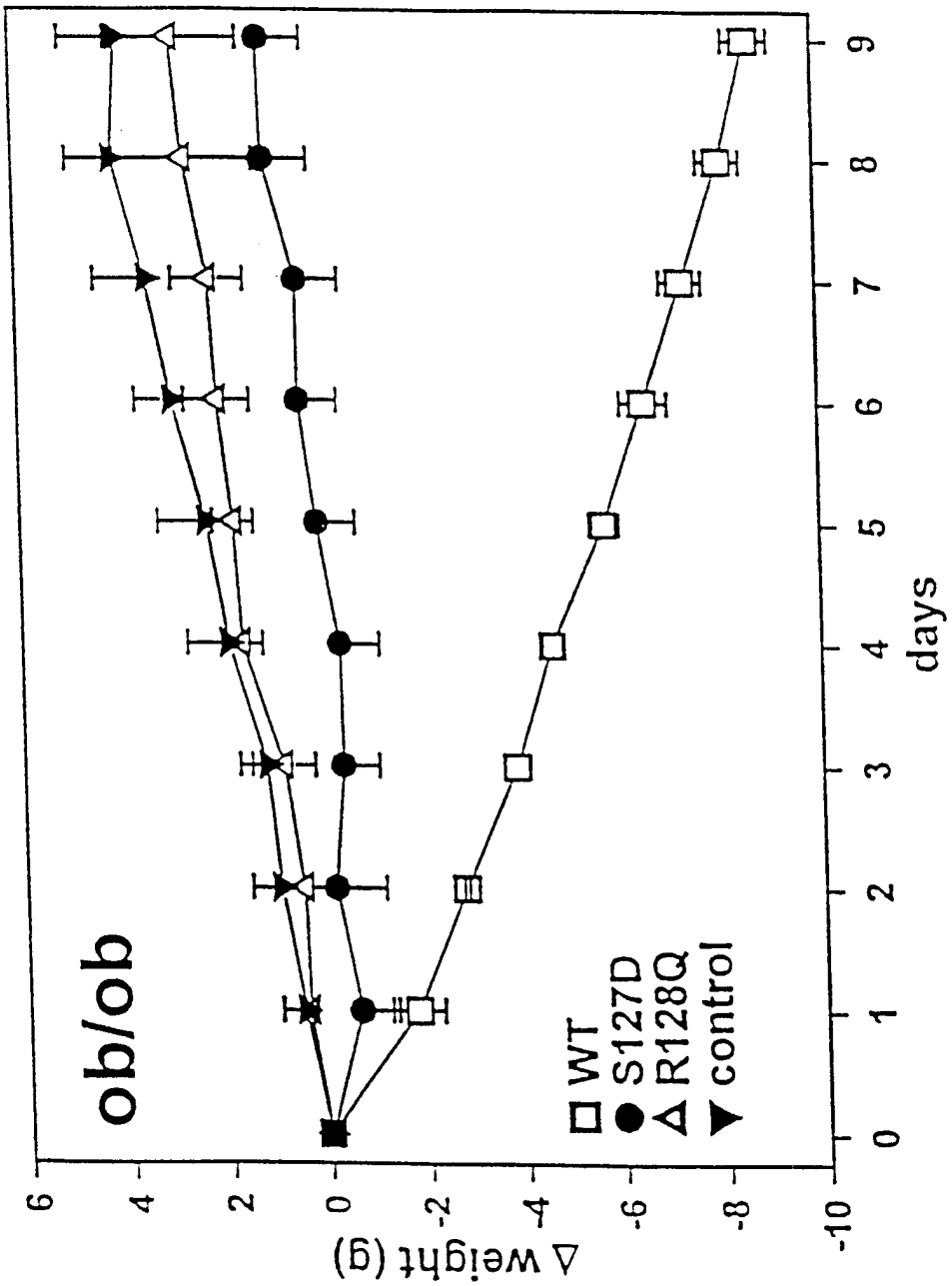
1/8
Fig. 1


bound (cpm 10^{-3})
12
10
8
6
4
2
0
0.001
0.010
0.100
1.000
10
[ob mutein] µg/ml
WT
R20Q
D40N
S127D
R128Q

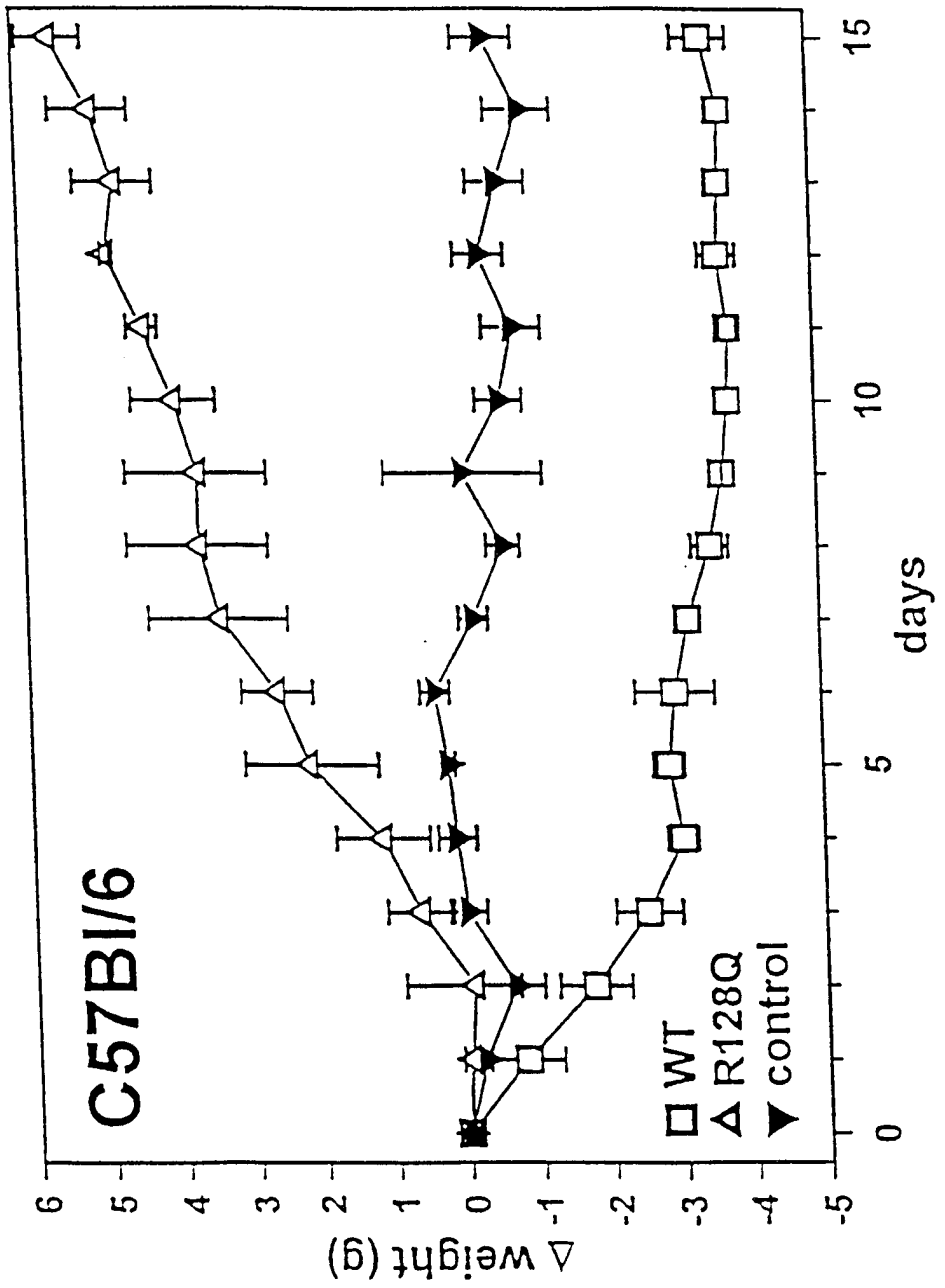
2/8
Fig. 2


WT
R20Q
D40N
S127D
R128Q
control
^{3}H TdR (cpm 10^{-3})
0
20
40
60
80
100
120
0.01
0.1
1
10
100
[ob mutein] nl/ml

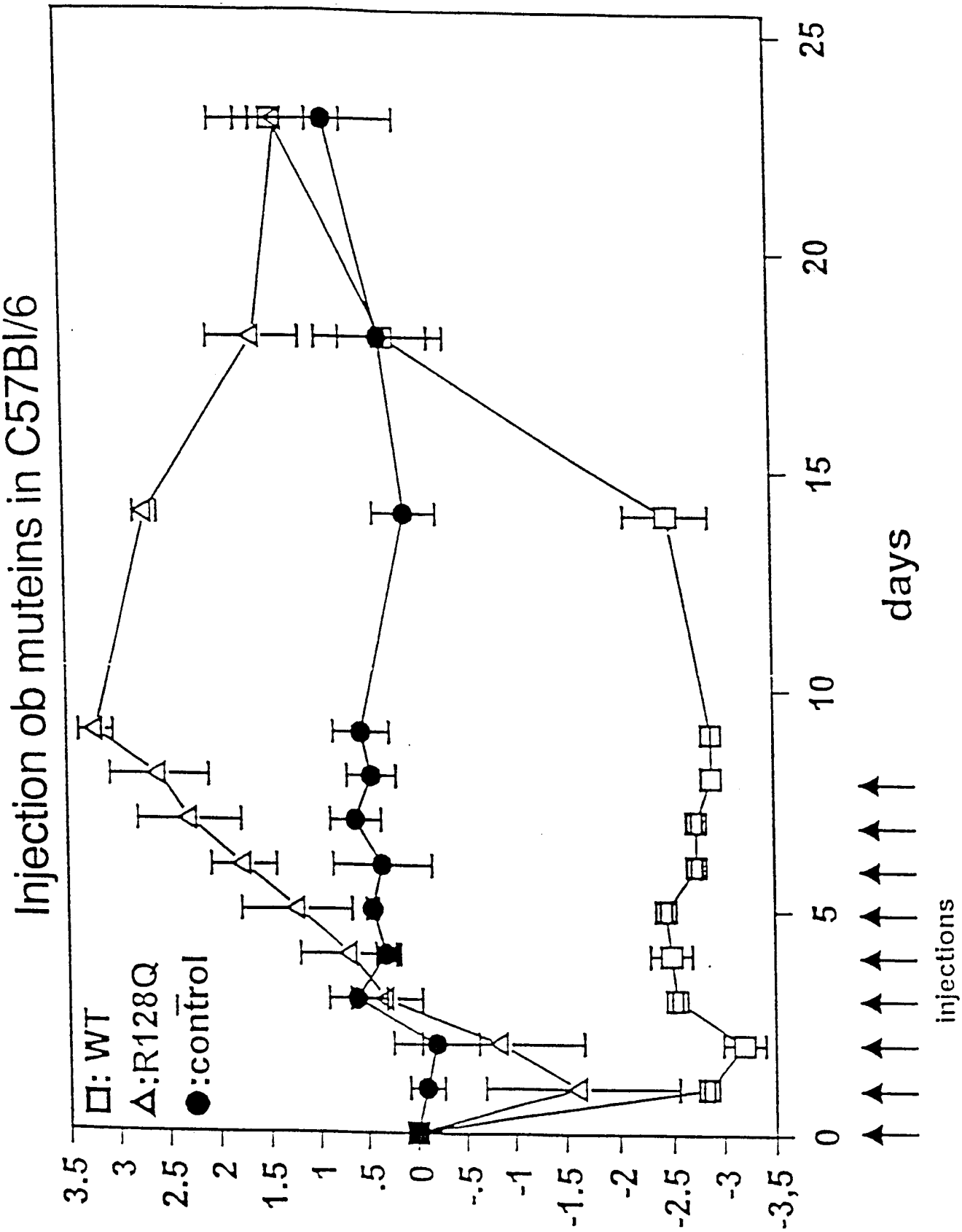
3/8
Fig. 3


ob/ob
WT
S127D
R128Q
control
Δ weight (g)
days
6
4
2
0
-2
-4
-6
-8
-10
0
1
2
3
4
5
6
7
8
9

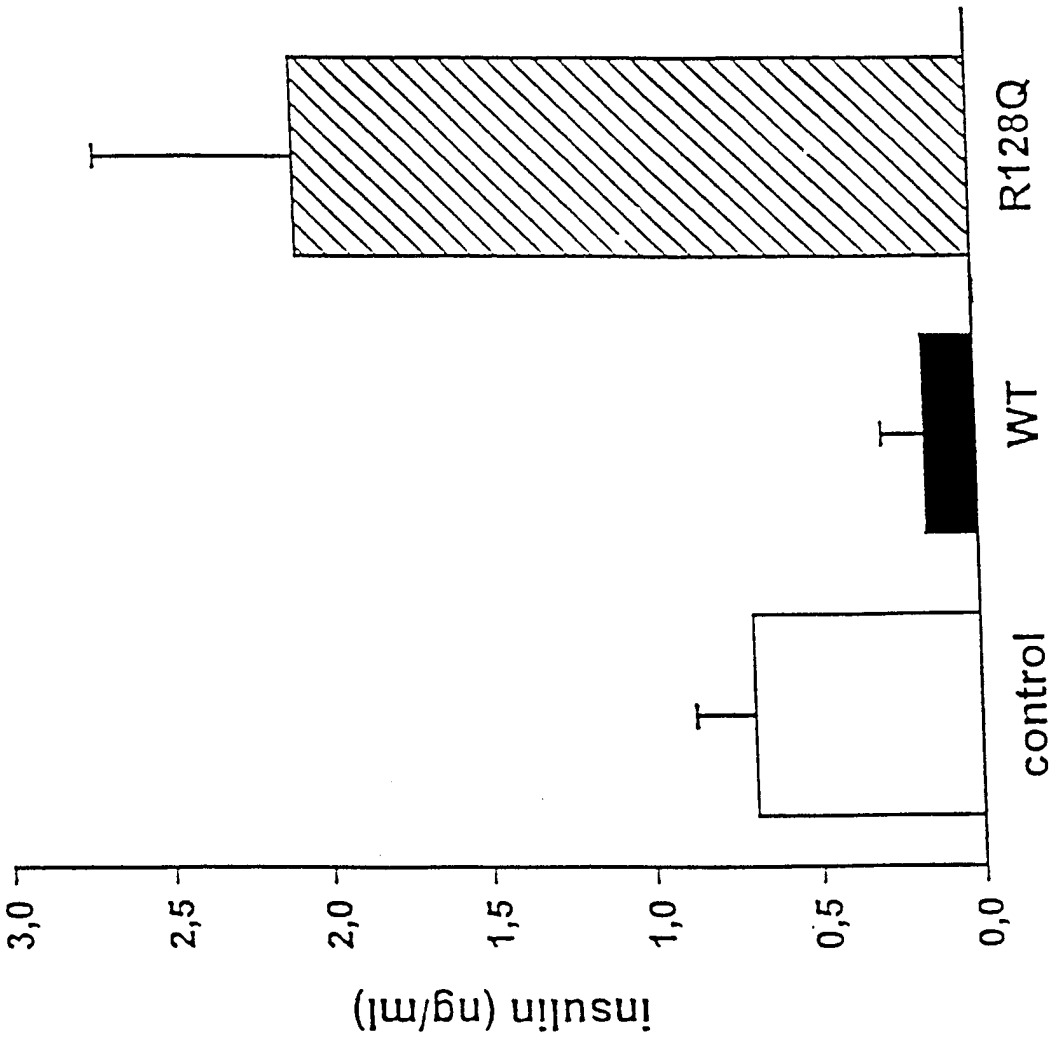
4/8
Fig. 4


C57Bl/6
Δ weight (g)
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
5
10
15
days
WT
R128Q
control

5/8
Fig. 5


Injection ob muteins in C57Bl/6
□: WT
Δ:R128Q
●:control
3.5
3
2.5
2
1.5
1
.5
0
-.5
-1
-1.5
-2
-2.5
-3
-3,5
0
5
10
15
20
25
days
injections

6/8
Fig. 6



7/8

Fig. 7

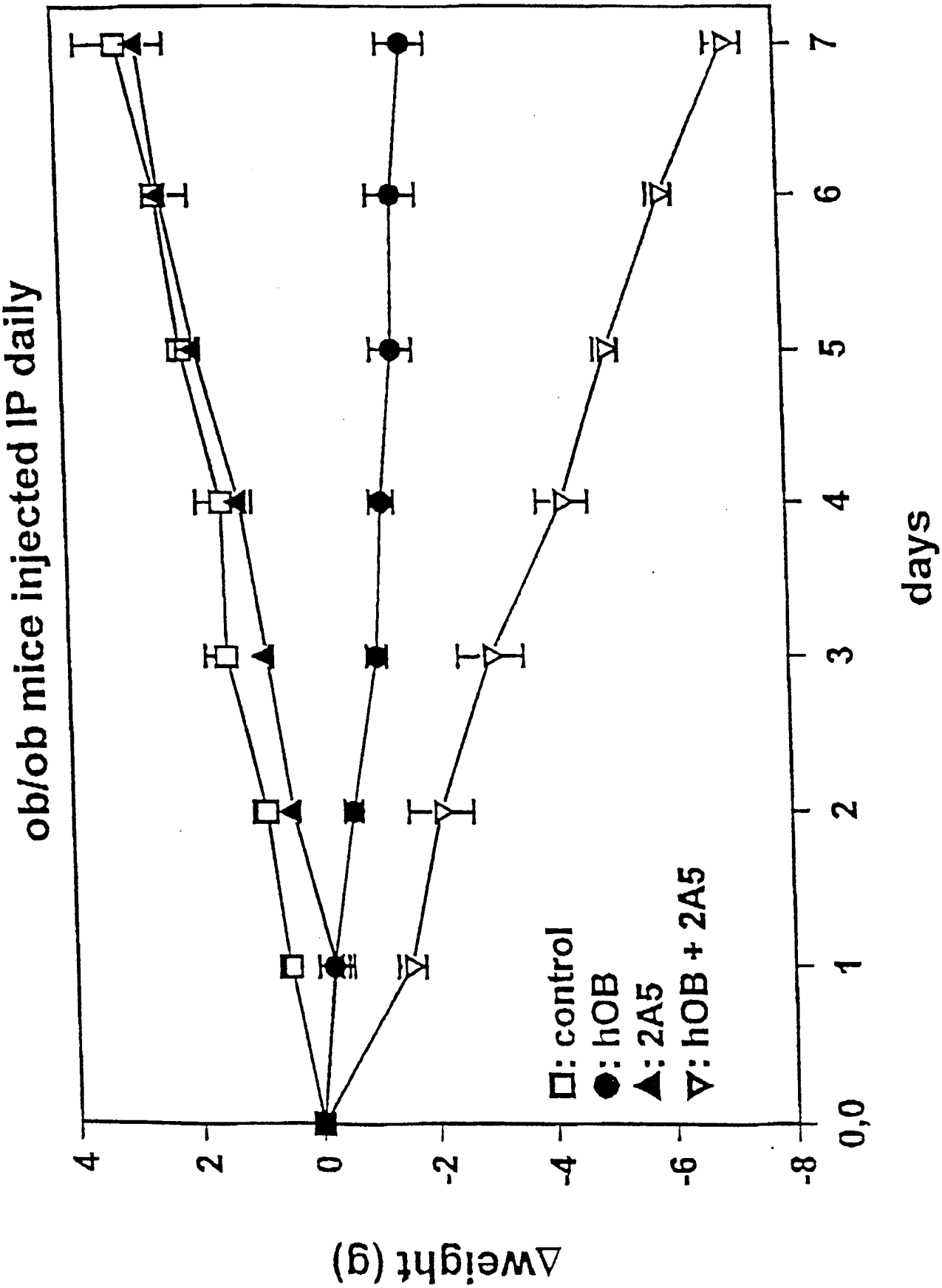

ob/ob mice injected IP daily
Δweight (g)
days
0,0
1
2
3
4
5
6
7
4
2
0
-2
-4
-6
-8
□: control
●: hOB
▲: 2A5
▽: hOB + 2A5

Fig. 8