The first international standard for human leptin and the first international standard for mouse leptin: comparison of candidate preparations by *in vitro* bioassays and immunoassays

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ABSTRACT

In an international collaborative study, two preparations of human sequence recombinant leptin and two preparations of mouse sequence recombinant leptin were evaluated, using *in vitro* bioassays and immunoassays, by eight laboratories, in three countries, for their suitability to serve as the international standard (IS) for human and mouse leptin respectively. The bioassays detected the human and mouse leptin with similar potency, while the immunoassays showed a greater response to the leptin of the species against which the antibody preparation had been raised. Comparison of the candidate standards with the various preparations of leptin of the same species currently assayed in the participating

laboratories showed that immunoassay measurements cannot be used to predict the biological potency. On the basis of the results reported here, in October 1999 the Expert Committee on Biological Standardization of the World Health Organization established the preparation coded 97/594 as the first IS for human leptin, with an assigned unitage of 4000 IU/ampoule, and the preparation coded 97/626 as the first IS for mouse leptin, with an assigned unitage of 4000 IU/ampoule. The ISs for leptin are distributed by the National Institute for Biological Standards and Control, UK, <http://www.nibsc.ac.uk>.

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INTRODUCTION

Leptin, also referred to as Ob, was identified (Zhang *et al.* 1994) as the product of the *ob* gene, a mutation which results in a morbid obesity in *obese* mice. It is synthesized as a 167-amino acid protein. Cleavage of the 21-amino acid signal sequence results in the 146-amino acid (M_r 16 000) nonglycosylated leptin molecule found circulating in the plasma (Cohen *et al.* 1996). The leptin sequence is highly homologous across a range of mammalian species (Zhang *et al.* 1997), with human and mouse leptin sharing 84% amino acid sequence identity. Splice variants of the leptin receptor have been identified (for a review, see Friedman & Halaas 1998), with only one form, Ob-Rb, having a cytoplasmic domain with all the

protein motifs necessary to activate fully signal transduction.

Leptin plays a central role in regulating the balance of fuel stores and energy expenditure, is active in the immune (Lord *et al.* 1998) and reproductive (Chehab *et al.* 1997) systems, and has angiogenic activity (Sierra-Honigmann *et al.* 1998). The role of leptin in the control of body weight is the subject of intensive research, and leptin, and modified forms of the leptin molecule, have been investigated for the treatment of obesity. Valid interlaboratory comparison of potency and immunoassay measurements of leptin samples requires the use of a common reference standard. The National Institute for Biological Standards and Control (NIBSC) therefore obtained and ampouled preparations of human and mouse leptin, the two

TABLE 1. Bioassays

	Cell type	Parameter measured	Lab. code	Assay code*
Assay type				
Cell metabolic activity	32D-expressing chimeric receptor with Ob-R extracellular domain	AlamarBlue reduction by absorbance	5	B5
		AlamarBlue reduction by fluorescence	8	B8h
Activated STAT-induced transcription	HEK-293-expressing Ob-Rb and luciferase under STAT-inducible promoter	Luciferase activity	2	B2h
			2	B2m

*B=bioassay; number=lab. code; h=human and m=mouse for IHS and form of leptin the assay is designed to measure.

most frequently measured species, and organized their evaluation by international collaborative study for their suitability to serve as the international standard for the respective species.

Aims of the study

The aims were to establish an international standard (IS) and the international unitage for human leptin and for mouse leptin by: (i) assessing the suitability of each of the candidate preparations of human and mouse leptin to serve as a standard in bioassays and immunoassays; (ii) comparing the candidate preparations with each other and with local standards; (iii) assessing the stability of each of the candidates by comparing them with ampoules of the same preparation subjected to accelerated thermal degradation; and (iv) estimating the nominal mass content of the ampoules for the purposes of calibrating immunoassays

Participants in the study

The following participants, listed alphabetically by country, contributed data to the study. In this report, each laboratory is identified by a number from 1 to 8 that is not related to this order of listing. Dr M W Elmlinger and Prof. Dr M B Ranke, Pediatric Endocrinology, Children's Hospital, Hoppe-Seyler-Str. 1, D-72076 Tuebingen, Germany; Dr A Janetzko, DRG Instruments GmbH, Frauenbergstr. 18, D-35039 Marburg, Germany; Dr J Kratzsch and Prof. Dr W Kiess, University of Leipzig, Department of Clinical Chemistry, Liebigstr. 27a, 04103 Leipzig, Germany and Department of Pediatrics, Oststr. 21-25, 04317 Leipzig, Germany, respectively; Dr L Pridzun, Mrs S Perrucci and Mr K-H Lindau, Mediagnost GmbH, Aspenhastr. 25, D-72770 Reutlingen, Germany; Mr B Rafferty, Mr R Stammers and Ms J Wickenden, NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, UK; Prof. P

Trayhurn and Miss J T Crabtree, Division of Biomedical Science, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK; Ms S Andrade and Ms J Schwanauer, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1789, USA; and Dr C I Rosenblum and Ms A Vongs, Merck Research Laboratories, PO Box 2000, RY80 M-213, Rahway, NJ 07065, USA.

MATERIALS AND METHODS

Two preparations of human sequence recombinant (rh) leptin and three preparations of mouse sequence recombinant (rm) leptin were donated to the World Health Organization (WHO) (see Acknowledgements). Potential excipients were tested for interference in an *in vitro* bioassay (Table 1, assay code B5) and two immunoassays (Table 2, assay codes E5h and E5m). Trial fills were conducted and the lyophilized leptin was compared with the bulk material and tested for its stability on storage at elevated temperatures. Two preparations each of rh and rm leptin, all synthesized in *E. coli*, were then ampouled at NIBSC, following Expert Committee on Biological Standardization (ECBS) guidelines (WHO 1990), and were coded as detailed in Table 3.

Buffer, final composition sodium citrate (10 mM pH 5.2), 2 mg/ml trehalose (Fluka Chemie AG, Buchs, Switzerland) and 5 mg/ml human serum albumin (Baxter Healthcare Corporation, Glendale, CA, USA), was prepared using nonpyrogenic water (Baxter Healthcare) and depyrogenated glassware. Before addition of the albumin, the solution was passed through a 0.22 µm APD membrane filter (Sartorius, Surrey, UK).

The concentrations of the leptin preparations received as frozen solutions were determined by the manufacturers by measuring the absorbance at 280 nm. On thawing, the solutions were clear, and were used without filtering. Approximately 10 mg

TABLE 2. Immunoassays

	First antibody/binding	Second antibody/detection	Lab. code	Assay code*	Reference
Assay type					
IFA	Solid phase rhOb-R extracellular domain, then anti-rh leptin (rabbit IgG)	Anti-rabbit IgG (goat)	1	F1h	Kratzsch <i>et al.</i> (1999)
ELISA	Anti-rh leptin (rabbit)	Anti-rh leptin (mouse monoclonal)	4	E4h	DRG Instruments GmbH, Germany, DRG EIA-2395 (leptin, sandwich)
	Anti-rh leptin (mouse monoclonal)	Anti-rh leptin (mouse monoclonal) biotinylated	5	E5h	In-house, unpublished
	Anti-rm leptin (rat monoclonal)	Anti-rm leptin (goat polyclonal) biotinylated	5	E5m	In-house, unpublished
	Anti-rh leptin (rabbit IgG)	Anti-rh leptin (rabbit IgG)	6	E6h	Hardie <i>et al.</i> (1997)
	Anti-rm leptin (rabbit IgG)	Anti-rm leptin (rabbit IgG)	6	E6m	Hardie <i>et al.</i> (1996)
RIA	Anti-rh leptin (rabbit IgG)	Anti-rabbit IgG (goat)	1	R1h	Kratzsch <i>et al.</i> (1999)
	Anti-rh leptin (rabbit Ig)	Anti-rabbit IgG	3	R3hs	Mediagnost GmbH, Germany, Human-Leptin-Sensitive-RIA, LEP-R40/41
	Anti-rh leptin (rabbit Ig)	Anti-rabbit IgG	3	R3h	Mediagnost, Human-Leptin-Standard-RIA, LEP-R42/43
	Anti-rh leptin (rabbit Ig)	Anti-rabbit IgG	7	R7hs	Mediagnost, Human-Leptin-Sensitive-RIA, LEP-R40/41
	Anti-rm leptin (rabbit Ig)	Anti-rabbit IgG	3	R3m	Mediagnost, Mouse/Rat-Leptin-RIA, LEP-R61
EIA	Anti-rh leptin (rabbit antiserum)	—	4	C4h	DRG Instruments, DRG EIA-1863 (leptin, competitive)

*The first character denotes the assay type (C=competitive EIA, E=ELISA (sandwich), F=functional immunoassay, R=RIA); the second character denotes the lab. code; the third character denotes the IHS, human (h) or mouse (m) (all recombinant), and the form of leptin, human or mouse, this assay is designed to measure and s denotes a high sensitivity version of an immunoassay.

TABLE 3. Leptin candidate standards

Ampoule code	Leptin type	Form received	Mean fill weight (g)	Weight range (g)	Residual moisture (%)	Predicted leptin per ampoule (µg)
97/594	Human	Solution (5 mg/ml)	1·0060	1·0044–1·0085	0·329	5
97/630	Human	Solution (2·6 mg/ml)	1·0046	1·0005–1·0088	0·376	3·8
97/626	Mouse	Solution (6·65 mg/ml)	1·0059	1·0027–1·0106	0·360	4·0
98/558	Mouse	Lyophilized	1·0079	1·0064–1·0092	0·199	4

of the preparation received as a lyophilized powder were dissolved in 3 ml PBS and filtered through a 0·2 µm low protein-binding sterile Acrodisc 13 (Gelman Sciences, Ann Arbor, MI, USA). The concentration of the solution was measured by absorbance and by Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, Herts, UK) using BSA standard. For each preparation, the appropriate volume was

added to the citrate buffer to give $3·6 \pm 1\%$ liters of a solution of concentration around 4 µg/ml leptin, and this solution was distributed in 1 ml aliquots, giving the theoretical leptin content per ampoule shown in Table 3.

The leptin content of the ampoules cannot be verified by direct measurement of absolute mass, so the content is assumed to be the theoretical mass,

calculated from the dilution of the bulk material of known leptin mass content. This value is described in units of 'predicted μg '. The leptin content of each batch of serum albumin was below the level of detection of the in-house immunoassay and could thus contribute a maximum of 2 parts per million of the total leptin content of an ampoule.

For each fill, 60–70 ampoules were weighed. The mean fill weights are shown in Table 3. Each solution was lyophilized, and the ampoules were sealed under dry nitrogen by heat fusion of the glass and stored at -20°C in the dark. Residual moisture of each preparation (mean of three ampoules), measured by the Karl–Fischer method, ranged from 0.20 to 0.38%.

The ampouled preparations were subjected to preliminary in-house testing to verify recovery of immunological reactivity and biological activity on lyophilization. Reconstituted ampoules were also subjected to cycles of freeze-thawing to verify that these preparations could be aliquoted on reconstitution, and frozen aliquots stored for later use.

Design of the study

Each participant received a selection of coded ampoules of the candidate standards and degradation samples, and labeled ampoules of lyophilized excipient. The degradation samples had been stored at 4°C , 20°C , 37°C and 45°C for periods of 3–13 months. Participants were asked to assay specified sets of ampoules, following their normal *in vitro* bioassay and immunoassay protocols, with two independent assays (each using freshly reconstituted ampoules) by each method, and to include their in-house standard (IHS), measuring responses at several dose levels with replicates for each sample. Pairs of samples, identical apart from their code (coded duplicates), were included to provide a direct measure of the intra-assay variation. To permit suitable dilution series to be made, participants were asked to assume a leptin content of $4\mu\text{g}$ /ampoule. To test for any effect of the lyophilization excipient, some participants were asked to assay their IHS in the presence of excipient, with the leptin:excipient ratio similar to that of the candidate standards. To test for the specificity of assays for human or mouse leptin, ampoules of both forms were included in some sets.

Bioassays and immunoassays

The test samples were examined in a variety of *in vitro* bioassays and immunoassays, as detailed in

Tables 1 and 2. The bioassays were based on the responses of two different genetically modified cell lines. One line (assay codes B5 and B8h) was a 32D transfectant expressing a chimeric receptor comprising the extracellular ligand-binding domain of the human leptin receptor, Ob-R, and the transmembrane and intracellular domains of the murine erythropoietin receptor (Crouse *et al.* 1998). The second line (assay codes B2h and B2m) was an HEK-293 transfectant expressing the signal-transducing form of the leptin receptor, Ob-Rb, and a STAT-inducible promoter regulating firefly luciferase cDNA (Rosenblum *et al.* 1998).

One laboratory used an immunofunctional assay (IFA) (assay code F1h) in which leptin was captured by immobilized recombinant extracellular, ligand-binding, domain of the human leptin receptor and the bound leptin was identified by rabbit IgG raised against rh leptin (Kratzsch *et al.* 1999).

Both commercial and in-house immunoassays were used for human and mouse leptin. Brief details and references are given in Table 2.

Statistical analysis

Data for each assay were examined both graphically and using analysis of variance (Gaines Das & Rice 1985), and outliers were omitted before further analysis. The majority of dose–response curves could be described using a four parameter logistic model. For these, asymptotes were determined and analysis of transformed responses was carried out using an in-house program, WRANL (Gaines Das & Tydeman 1982), which provides weighted regression analysis of logit response on log dose with an assessment of linearity and parallelism, and estimates of relative potency. One laboratory (code 6) assayed test samples at a single dose and potencies for these were determined assuming all preparations would have given dose–response lines parallel to the IHS.

For calculation of the relative potencies, the theoretical ampoule contents were corrected from the $4\mu\text{g}$ leptin/ampoule assumed by the participants to the predicted values detailed in Table 3. Estimates of relative potency were combined as geometric means and confidence limits were determined using variance of the log potency estimates. The geometric coefficient of variation (GCV, determined as $\exp(s) - 1$, multiplied by 100 to give percent, where s is the standard deviation of the log potency estimates) has been used to provide a measure of the precision of estimates.

TABLE 4. Potency of candidate standards: laboratory and method geometric mean estimates for human leptin, 97/594 and 97/630, and mouse leptin, 97/626 and 98/558, relative to the various IHSs, expressed as μg IHS per predicted μg ampouled preparation, and of 97/630 relative to 97/594, 97/626 relative to 97/594, 98/558 relative to 97/594 and of 98/558 relative to 97/626. Estimates are based on direct within-assay comparisons

Assay code*	97/594 (human)	97/630 (human)	97/626 (mouse)	98/558 (mouse)	97/630:97/594	97/626:97/594	98/558:97/594	98/558:97/626
B2h	0.76	0.68	—	—	0.85	—	—	—
B2m	0.79	—	0.86	0.84	—	1.05	—	0.93
B5	No IHS	—	—	—	0.41	1.44	1.05	0.73
B8h	0.64	0.42	0.78	0.59	0.66	1.11	0.81	0.76
E4h	1.06	0.65	—	—	0.61	—	—	—
E5h	No IHS	—	—	—	0.42	0.18	—	—
E5m	No IHS	—	—	—	—	9.78	5.68	0.58
E6h	0.95	1.14	—	—	1.20	—	—	—
E6m	—	—	0.96	0.57	—	—	—	0.60
R1h	1.01	0.79	—	—	0.78	—	—	—
R3h	1.06	0.74	—	—	0.70	—	—	—
R3hs	1.07	0.74	—	—	0.69	—	—	—
R3m	—	—	1.43	0.95	—	—	—	0.67
R7hs	1.15	0.81	0.20	0.30	0.70	0.18	0.26	1.46
C4h	1.09	0.80	—	—	0.74	—	—	—
F1h	1.35	0.76	—	—	0.56	—	—	—

*Detailed in Tables 1 and 2.

RESULTS

The test samples were examined in *in vitro* bioassays, immunoassays and a ligand IFA (a total of 57 assays), as detailed in Tables 1 and 2. The leptin candidate standards and IHS of the same species behaved similarly to each other; the dose–response lines were generally found to be linear and parallel. Fewer than 0.1% of the responses were deleted as outliers. In the assay R7hs for human leptin, the two mouse leptin candidate standards gave responses near the end of the range, so a single dose was used to calculate approximate potencies, and the data were not used for any other calculations.

Assay accuracy and precision assessed using coded duplicates

Under ideal conditions, coded duplicate preparations would have identical activities, and the deviation of the relative potency from the value of 1 reflects both the accuracy and precision of the assay. Comparison of the first of each pair of coded duplicates with the second gave values close to the expected relative potency of 1; the overall geometric mean of assay means for each pair was 1.05 (95% limits: 0.99–1.09).

Within assays, the deviation of the relative potency of the duplicates from the value 1 shows the 95% limits for an individual potency estimate would

be 70–143% (GCV 20%). Between assays, within a laboratory, the variability was similar (GCV 18%). This is typical of the precision seen for many *in vitro* assays (Robinson *et al.* 1998, Gaines Das *et al.* 1999).

Comparison of the candidate standards with each other and with the IHSs

Estimates of the relative potency of the candidate standards in terms of the various IHSs are summarized in Table 4.

Estimates by immunoassays of the human leptin per ampoule in terms of the various human leptin IHSs are consistent with the predicted value of 5 μg for 97/594 and 4 μg for 97/630. Similarly, the estimates by immunoassays of the mouse leptin per ampoule in terms of mouse leptin IHSs are consistent with the predicted value of 4 μg for 97/626 and 4 μg for 98/558. Both human and mouse immunoassays give higher measurements for leptin of the appropriate species than of the other species.

Comparison of the candidate standards and the IHSs of the same species shows that the ratio of biological to immunological activity varies between preparations. Preparation 97/594, for example, shows a lower ratio of biological to immunological activity than do the various human IHSs, as illustrated by the distribution of potency estimates by the different assay systems, shown in Fig. 1.

FIGURE 1. Potency of preparation 97/594, first IS for leptin, relative to the various human leptin IHSs, expressed as μg IHS per predicted μg of 97/594. Each square represents an independent assay and the designation in the square is the assay code as shown in Tables 1 and 2. The distribution of estimates shows that 97/594 has a higher ratio of immunological to biological activity than do the various IHSs.

Comparisons of the two human leptin candidate standards with each other and of the two ampouled mouse leptin preparations with each other gave estimates which were broadly consistent between laboratories. The overall geometric means of laboratory mean estimates were 0.68 predicted μg of 97/594 equivalent to 1 predicted μg of 97/630 (GCV 35%) and 0.77 predicted μg of 96/626 equivalent to 1 predicted μg of 98/558 (GCV 39%). In contrast, the between-laboratory variability for comparison of either human preparation with either mouse preparation was substantially larger with, in each case, GCV greater than 200%.

Bioassay comparison of mouse and human leptins was limited to two or three laboratories. Estimates for comparison of either mouse leptin with human leptin 97/594 (mean relative potencies 1.05, 1.42, 1.11 for 97/626 and 1.04, 0.84 for 98/558) showed better agreement than estimates for comparison of either mouse leptin with human leptin 97/630 (relative potencies 3.51, 1.72 for 97/626 and 2.57, 1.25 for 98/558).

Effect of the lyophilization excipient

The activity of the participants' IHS preparations of leptin, assayed in the normal manner, was compared

with the activity of the IHS assayed in the presence of the excipient used in the preparation of the candidate standards (at a leptin:excipient ratio similar to that in the candidate standards). The excipient did not appear to have any effect; the geometric mean of the ratio of activity of the IHS to activity in the presence of excipient was 1.01 (95% limits: 0.95–1.07).

Stability

Stability of the candidate standards was assessed using thermally degraded samples. For each preparation there was a tendency for samples which had been stored at +45 °C to have slightly lower potencies than the samples stored continuously at –20 °C, but these differences were not significant compared with the assay variability as assessed by the coded duplicates. Samples stored at lower temperatures showed no evidence of decreased potency, as shown in Table 5.

DISCUSSION

For the preparations of human leptin, as for the preparations of mouse leptin, the dose–response

TABLE 5. Stability of candidate standards. Geometric mean of all individual estimates, with 95% confidence intervals, for thermally accelerated degradation samples, calculated ignoring differences in assay methods and in storage times for some samples. The minimum time of storage at any of the elevated temperatures has been given, although in several cases samples were stored at some temperatures for longer times

	Minimum storage (days)	Storage temperature (°C)				
		− 20	4	20	37	45
Leptin						
97/594	349	1.00 (0.90–1.11)	1.04 (0.94–1.16)	1.10 (0.98–1.24)	0.94 (0.85–1.05)	0.92 (0.83–1.02)
97/630	168	1.01 (0.95–1.08)	1.00 (0.95–1.05)	0.98 (0.90–1.07)	0.90 (0.82–1.00)	0.84 (0.69–1.02)
97/626	294	1.05 (0.91–1.22)	1.07 (0.89–1.29)	1.06 (0.84–1.33)	1.05 (0.89–1.22)	0.98 (0.55–1.72)
98/558	92	1.03 (0.99–1.07)	1.04 (0.97–1.10)	0.90 (0.68–1.19)	1.05 (0.95–1.15)	0.91 (0.81–1.01)

curves of the two candidate standards and the various IHSs are parallel, so valid comparisons of potency can be made between the various human preparations and between the various mouse preparations. Interlaboratory variation in estimates of leptin potency is reduced by use of a common standard in place of various IHSs.

The bioassays detect the human and mouse leptin with similar potency. The bioassays are all based on cell lines which express either the long form of the human leptin receptor or the extracellular domain of the human leptin receptor in a chimeric molecule. This cross-species activity is similar to the situation *in vivo* where human leptin is biologically active in mice (Verploegen *et al.* 1997). In contrast, the immunoassays in which both human and mouse leptin were tested showed a much greater response to the leptin species against which the antibody preparation had been raised.

Comparison of the two human leptin candidate standards with each other and with the various IHSs, in assays designed to measure human leptin, gave generally consistent relative potencies across the bioassays and across the immunoassays, with 97/630 generally less potent than 97/594. However, there is a difference between the bioassay and immunoassay results, with both candidates showing a lower biological to immunological potency ratio than the IHSs. Similar comparison of the mouse leptin preparations in assays designed for mouse leptin, shows consistent relative potencies across the bioassays and across the immunoassays, but comparison of bioassay and immunoassay data shows that 97/626 is relatively more biologically active than 97/558. These results illustrate the general principle that immunoassay measurements cannot be used to predict biological potency.

The excipient in which the four candidate standards were lyophilized had no detected effect on any of the assay systems in which it was tested, so each of the candidates could be used as a standard

without any requirement to compensate for interference from the excipient.

All of the candidate standards were judged, from the accelerated thermal degradation results, to be sufficiently stable to serve as a standard. Although there was a tendency for samples which had been stored at +45 °C to show a small decrease in activity compared with the samples stored continuously at –20 °C, this difference was not judged significant when compared with the variation in estimates for the coded duplicates. Samples stored at lower temperatures showed no decrease in potency.

On the basis of the consistency of the estimates of potency and predicted stability on storage, both of the human leptin candidates and both of the mouse leptin candidates are suitable to serve as standards for the relevant form of leptin. For both human and mouse leptin, it was proposed that the candidate for which there is longer-term degradation data and which shows the higher potency be selected as the first IS.

With the agreement of the participants in the collaborative study, at its 50th meeting in Geneva, in 1999, the ECBS of WHO established preparation 97/594 as the first IS for human leptin, with an assigned unitage of 4000 IU/ampoule and preparation 97/626 as the first IS for mouse leptin, with an assigned unitage of 4000 IU/ampoule, with the statements that for the purpose of immunoassay calibration, the nominal human leptin content of 97/594 may be assumed to be 5 µg/ampoule and the nominal mouse leptin content of 97/626 may be assumed to be 4 µg/ampoule, and that these data should not be assumed to validate the use of immunoassay for the control of potency of therapeutic preparations of leptin. The IS may be obtained for use in the calibration of local standards by writing to NIBSC, PO Box 1193, Potters Bar, EN6 3QH, UK, or through Web site <http://www.nibsc.ac.uk>.

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