Report

SEVENTH WHO INFORMAL CONSULTATION ON STANDARDS FOR CYTOKINES, GROWTH FACTORS AND ENDOCRINOLOGICAL SUBSTANCES

The National Institute for Biological Standards and Control (NIBSC), UK, 20-21 October 2003

WORLD HEALTH ORGANIZATION
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Introduction

The seventh WHO Informal Consultation on Standards for Cytokines, Growth Factors and Endocrinological Substances was held at The National Institute for Biological Standards and Control (NIBSC) on 20-21 October 2003. Several issues relating to biological standardization and the establishment of new reference materials were discussed. In particular, recent regulatory requirements within the European Union, which have implications for potency assignments for WHO international standards (IS) and other biological reference materials, were discussed. As a priority, the data on candidate reference materials proposed to the November 2003 WHO Expert Committee on Biological Standardization (ECBS) as proposals to the ECBS to establish as IS or reference reagents (RR) were reviewed. Candidate reference materials, including those for human interferon beta (IFN-β), human tumour necrosis factor alpha (TNF-α), human thyroid stimulating hormone (TSH) and human luteinizing hormone (LH) were discussed and analyzed in detail. In addition, the immunogenicity of therapeutic proteins, including interferons, cytokines and hormones, together with a specific proposal to adopt a standard method for the calculation and reporting of results of interferon neutralizing antibody tests were reviewed and discussed. Lastly, the on-going cytokine and hormone standardization programme at NIBSC was reviewed.


Recent, legally enforceable, requirements from the International Organization on Standards (ISO), published in the document prEN/ISO 17511 and adopted in the European Union (EU) In Vitro Diagnostics Directive, itemized approaches to traceability and stability of reference materials used to support the manufacture and use of diagnostics. The ISO 17511 requirements for reference standards specify assignment of SI units to ampoule content by single (reference) method studies and traceability to a previous standard, with defined uncertainty limits. Such requirements are presently contrary to the acceptance criteria for WHO IS (WHO 1990)\(^1\). The WHO IS contain biological materials that cannot be fully characterized by chemical and physical methods alone and whose activity is evaluated by multi-method studies and assigned in International Units (IU) per ampoule, with no imprecision given for ampoule content. The assigned IU is universally applicable for the calibration of secondary (regional, national, in-house working) standards containing the same/similar material(s) and bioassays for measurement of relative activity of test samples of the same/similar material(s). Since adoption of ISO 17511 requirements poses serious concerns for some users of WHO IS (especially in the diagnostic area), who are now required to express traceability and uncertainty of reference materials, WHO has instituted a review of its Guidelines on IS (WHO 1990)\(^1\) to address these issues.

Following discussion of the issues, it was concluded that:

- further discussion was needed to inform the ISO of the difficulties in applying ISO 17511 requirements to biological reference materials.
• the WHO to publish an article as soon as possible in one of its scientific journals on its acceptance criteria for biological reference preparations.
• a WHO Working Group should formulate/revise the guidelines and take the matter forward. As a first step, it was suggested that the memoranda accompanying the dispatch of the IS should include the uncertainty limits of fill variation in ampoules of IS together with available information on assessment of stability. It was recommended these steps be notified to ECBS 2003 for consideration.

Nomenclature
Inconsistencies regarding the naming of WHO IS were noted. No specific recommendations were made, except that such inconsistencies should be addressed in the revision of the WHO Guidelines such that unique identification is maintained.

Stability of reference materials
While it was accepted that in principle long-term or “real time” stability testing is desirable, it was recognized that application to all current WHO IS and RR would generate an unacceptably high workload. Therefore, it was recommended that:-

• long-term stability testing should be implemented on a case-by-case basis following recommendations from ECBS about certain IS/RR.
• regular report back on long term stability testing should be provided to the ECBS, in the appropriate timeframe.
• an appropriate number of ampoules of every new reference preparation should be stored at -150 C to provide a baseline for activity determinations.
• if possible, stability data should be collected from manufacturers who use WHO IS/RR in order to provide assurance of stability under routine laboratory conditions.

Session 2: Review of the data on candidate materials proposed to the ECBS 2003 for establishment as International Standards or Reference Reagents

Interferon beta
The current 2nd WHO IS of IFN-β, Gb23-902-531, was prepared from partially purified IFN-β derived from human fibroblasts. The IFN-β was approximately 1% pure, and the preparation has subsequently been shown to contain cytokines other than IFN-β, most notably interleukin-6 (IL-6) (Meager, unpublished results). Some investigators have reported that inconsistent potency estimates of test IFN-β preparations may result when this IS was used to calibrate biological assays (bioassays) and the relative potencies of in-house 'working' standards, particularly when different bioassays were compared, suggesting undesirable influence of cytokine contaminants. The Informal WHO Consultative Group on Cytokine Standardization considered this issue at its 3rd meeting (NIBSC, 15-16th May 1997). It recommended that an international collaborative study be organized to address this issue and that both existing WHO IS and newly lyophilized candidate international standards (CIS) prepared at NIBSC should be examined in the study. NIBSC was requested and agreed to undertake the organization of the study. The present Consultative Group was informed about the completion of a recent WHO international study to evaluate existing IS and new CIS of human interferon-beta.
(IFN-β) aimed at assessing the current status of the biological standardization IFN-β. Data from bioassays carried out by 16 participating laboratories from 8 countries were received and statistically analyzed. Among the preparations studied, CIS containing CHO cell- or human fibroblast-derived, highly purified, IFN-β gave similar low variation in potency estimates one to another as the coded internal duplicates, which was significantly less than to the current 2nd WHO IS of IFN-β, human fibroblast-derived, Gb23-902-531. Consistent with all bioassay types, CIS designated 00/572, containing CHO cell-derived IFN-β and formulated with both human serum albumin and bovine casein for high recovery and stability, could be assigned a potency of 40,000 IU per ampoule relative to the IU of the current 2nd IS of IFN-β, Gb23-902-531. This assignment of 40,000 IU per ampoule was consistent and continuous with the relative activities of all other CIS that contained glycosylated CHO cell-derived or human fibroblast-derived IFN-β, but not with those of preparations included in the study that contained non-glycosylated, recombinant E. coli-derived IFN-β Ser 17 mutein (an IFN-β analogue in which the cysteine at position 17 is substituted by a serine residue).

Based on the data analysis of the WHO international collaborative study and the desirability of replacement of the 2nd IS of IFN-β, human fibroblast-derived, Gb23-902-531, because of its known cytokine contamination, it was recommended to the ECBS that:-

- CIS 00/572 containing CHO cell-derived human IFN-β, which is shown suitable to serve as an IS for both CHO cell-derived and human fibroblast derived IFN-β with an assigned potency of 40,000 IU per ampoule, replaces the 2nd IS of IFN-β, human fibroblast-derived, Gb23-902-531.
- CIS 00/572 is established by ECBS as the 3rd IS of glycosylated human IFN-β and that the 2nd IS of IFN-β, human fibroblast-derived, Gb23-902-531, is discontinued.
- Establishment of 00/572 as the 3rd IS to be subject to the provision to ECBS of additional specifications of the purified IFN-β that was used in the preparation of 00/572.

As 00/572 is suitable for the standardization of all glycosylated human IFN-β preparations but not non-glycosylated, recombinant E. coli-derived IFN-β Ser 17 mutein, it was further recommended to the ECBS that:-

- the 1st IS of IFN-β Ser 17 mutein, Gxb02-901-535, continue to serve as the IS for standardization of IFN-β Ser 17 mutein and other non-glycosylated IFN-β preparations whose dose-response curves have the same slope as this IS.
- The memorandum accompanying dispatch of the 1st IS of IFN-β Ser 17 mutein, Gxb02-901-535, state that it is not suitable for the standardization of glycosylated forms of human IFN-β. Conversely, the memorandum accompanying dispatch of 00/572, the proposed 3rd IS of glycosylated human IFN-β, state that it is not suitable for the standardization of IFN-β Ser 17 mutein.

**Tumour necrosis factor-alpha**
The 1\textsuperscript{st} IS for human tumour necrosis factor-alpha (hTNF-\(\alpha\)), 87/650, was established by ECBS in 1991. Since then there has been a high demand for 87/650 and by July 2003 stock numbers of ampoules had fallen to 250. It is vital to conserve this remaining stock of 87/650 for any future standardization and traceability purposes, e.g., for use in collaborative studies to evaluate future candidate IS of hTNF-\(\alpha\). Therefore, replacement of 87/650 is now urgent. Since there are however large stocks of three CIS of hTNF-\(\alpha\), 88/782, 88/784, and 88/786, which were prepared about 18 months after 87/650 and were included in the 1\textsuperscript{st} WHO international collaborative study to evaluate such reference preparations (Meager & Gaines Das) \textsuperscript{2} it is proposed that since replacement of the IS is urgent and preparation and evaluation of new CIS would be time-consuming, one of the three available well-characterized CIS should be chosen as the replacement of 87/650. Candidate IS 88/786 contains 1.0 \(\mu\)g of full length, 157 amino acid, hTNF-\(\alpha\) and, although sourced differently (Fukuda \textit{et al}) \textsuperscript{3}, is the most similar of the three candidate IS to the present 1\textsuperscript{st} IS 87/650.

The Consultative Group having been informed of this situation and the results of recent accelerated thermal degradation studies that confirmed the existing CIS would be suitable to serve as IS, recommended to the ECBS that:

- CIS 88/786 containing natural hTNF-\(\alpha\) derived from human lymphoblastoid BALL-1 cells (Fukuda \textit{et al}) \textsuperscript{3}, replace the 1\textsuperscript{st} IS 87/650 and be established as the 2\textsuperscript{nd} IS of hTNF-\(\alpha\).
- a potency of 46,500 IU per ampoule, the relative activity compared with that of 87/650 calculated on the basis of the results deriving from a reference bioassay in the international collaborative study \textsuperscript{2}, be assigned to 88/786.
- the establishment of 88/786 as the 2\textsuperscript{nd} IS of hTNF-\(\alpha\) to be dependent on the provision to ECBS of additional specifications of the purified BALL-1 cell-derived hTNF-\(\alpha\) that was used in the preparation of 88/786.
- the memorandum that accompanies dispatch of 88/786 states that users may experience discontinuities with the unitage/nominal content of the 1\textsuperscript{st} IS, 87/650, if 88/786 is used for the calibration of bioassays to measure the potency of anti-hTNF-\(\alpha\) materials or for the calibration of immunoassays to determine hTNF-\(\alpha\) concentration.

Since the suitability of 88/786 to serve as a bioassay calibrant for measuring the potency of anti-hTNF-\(\alpha\) products has not been established, it was further recommended that:

- collaborative studies to examine the compatibility of this proposed 2nd IS in anti-hTNF\(\alpha\) bioassays be carried out.

**Thyroid stimulating hormone**

Imunoassays for thyroid-stimulating hormone (TSH) are widely applied in the diagnosis and management of thyroid dysfunction. The calibrators in these assays are secondary standards whose activities have been assigned relative to a primary standard, typically the 2\textsuperscript{nd} IRP for TSH, Human, for Immunoassay, 80/558 (Gaines Das & Bristow 1985) \textsuperscript{4}. Stocks of this preparation are depleted and a replacement standard is now required.
In the original study in which the 2\textsuperscript{nd} IRP was established, three other ampouled pituitary TSH preparations were calibrated in terms of the 2\textsuperscript{nd} IRP, two of which, 81/565 and 81/615, were derived from the same batch of pituitary material as the IRP.\textsuperscript{4} It was anticipated that the preparation of TSH, 81/565, would be suitable to serve as an International Reference Reagent for immunoassay subject to confirmation of its original relative activity and stability. The third preparation of TSH, 81/502, was derived from a different pituitary source but was formulated and filled similarly to the above-mentioned materials.\textsuperscript{4}

The Consultative Group, having been informed of the results of an international collaborative study in which the TSH preparations described above were evaluated as CIS for the replacement of 80/558, recommended to the ECBS that:-

- the CIS 81/565 be established as the 3\textsuperscript{rd} IS for TSH for immunoassay standardization with an assigned potency of 11.5 mIU per ampoule.

**Recombinant human luteinizing hormone**

Luteinizing hormone (LH) activity, mostly as human chorionic gonadotrophin (hCG), is used widely, in combination with follicle stimulating hormone (FSH), for the treatment of infertility in women, and sometimes also in men. Until recently, the only form of LH available for treatment purposes has been human menopausal gonadotrophin. This consists of a purified extract of the urine of post-menopausal women, and contains both FSH and LH. However, recently, recombinant human LH (lutropin alfa; rLH) became licensed for therapeutic use. Therefore, a reference preparation of rLH, 96/602, was prepared and proposed to serve as the IS for rLH. 96/602 was evaluated by international collaborative study in comparison to two other rLH preparations 96/816 and 96/820 (prepared in the same way as the proposed IS and from the same rLH preparation), the 4\textsuperscript{th} IS for Human Urinary FSH and LH, 98/704, and the 2\textsuperscript{nd} IS for Human Pituitary LH, 80/552 (Storring & Gaines Das).\textsuperscript{5}

The Consultative Group, having been informed of the results of an international collaborative study in which the reference preparations described above were evaluated, recommended to the ECBS that:-

- the proposed IS of rLH, 96/602, be established as the 1\textsuperscript{st} IS of rLH and assigned a potency of 189 IU to maintain continuity with the IU of the 4\textsuperscript{th} IS for Human Urinary FSH and LH, 98/704.

**Review of the data for potency re-assignment of the 2\textsuperscript{nd} IS of human lymphoblastoid interferon alpha n1, 95/568 (established in 1999)**

Following the establishment of the 2\textsuperscript{nd} IS of human lymphoblastoid IFN-\textalpha n1, 95/568, with assigned potency of 38,000 IU per ampoule (Meager \textit{et al})\textsuperscript{6}, extensive studies performed in Japan have shown a discrepancy between the IU of 95/568 and that of J-501, the Japanese National Standard. Originally, J-501 was assigned 6,500 IU on the basis of results derived from bioassays calibrated with the 1\textsuperscript{st} IS of human

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lymphoblastoid IFN-α n1, Ga23-901-532. However, recent studies have indicated that the assignment of 6,500 IU to J-501 was incorrect and this should have been 5,500 IU. All clinical lots of human lymphoblastoid IFN-α n1 manufactured in Japan have been assigned potencies based on the original assignment of 6,500 IU to J-501. Therefore to maintain continuity with the IU of 95/568 and avoid changing the potencies assigned to clinical lots, it has been proposed that the potency of 95/568 is changed to 50,000 IU, the potency assignment that is necessary for continuity with 6,500 IU assigned to J-501.

The Consultative Group, having been informed of the results of the Japanese Studies in which J-501 was compared with both the 1st and 2nd IS of human lymphoblastoid IFN-α n1, could find no scientific basis to propose to ECBS that the potency of the 2nd IS, 95/568, be re-assigned to 50,000 IU. This proposal was thought to set the undesirable precedent of re-assigning a potency of an IS relative to that of a National Standard. It was recognized that maintaining the currently assigned potency of 38,000 IU to 95/568 would have adverse implications on the use of human lymphoblastoid IFN-α n1 produced and used in Japan. However, potential solutions should be further explored taking into account a problem that other users, including manufacturers of human lymphoblastoid IFNα-n1 may face. Until this information is available, it is premature to discuss this matter. Therefore, it was recommended that:-

- the issue should not be included in the Agenda for the ECBS in November 2003.
- following investigation of the usage of the 2nd IS, 95/568, and of the impact of a potency re-assignment to any existing and new potential manufacturers of human lymphoblastoid IFN-α n1 outside of Japan, the options regarding this issue should be reviewed.

Session 3: Therapy-induced antibodies against therapeutic proteins

A comprehensive review of the subject of immunogenicity of therapeutic proteins was presented highlighting the problems associated with the detection and quantification of therapy-induced antibodies and disease-associated autoantibodies by immunoassays and bioassays. The lack of appropriate reference antibody preparations in many cases and the use of non-standardized assay methods together with differing ways of calculating antibody concentrations have led to wide disparities both in the reporting of results and in the interpretation of results regarding clinical relevance. The Consultative Group recognized that prediction of immunogenicity of therapeutic proteins and accurate evaluation of therapy-induced antibodies was crucial to assessing the clinical safety of these proteins. It was recommended that:-

- the WHO become more involved in the “immunogenicity area”.
- to encourage progress towards better understanding of immunogenicity and the performance and analysis of quantitative assays to measure therapy-induced antibodies, the WHO to co-sponsor Workshops on ‘Animal models to investigate immunogenicity’ and on ‘Assays for quantification of binding and neutralizing therapy-induced antibodies’, respectively.
• the WHO initiate the drafting of Guidelines on non-clinical and clinical approaches to predicting immunogenicity and detection and measurement of therapy-induced antibodies.

• the WHO to review therapeutic proteins on a case-by-case basis and prioritize needs for appropriate reference antibody preparations and investigative studies for monitoring the development of therapy-induced antibodies. It was agreed that provision of human sera containing defined and characterized antibodies against cytokines (as can be obtained from patients undergoing therapy) would be very valuable. Provision of antiglobulin reagents for use in immunogenicity assays was also discussed, but some problems such as the issue of commercial conflict were recognized as being specifically associated with this.

A recommendation from the Standards Committee of the International Society for Interferon and Cytokine Research (ISICR) to adopt a standard method for the calculation and reporting of the results of interferon neutralizing antibody tests.

Interferon (IFN)-alpha and -beta products have been found to be immunogenic in some patients. A variable proportion of affected patients develop neutralizing antibodies (NAbs) to the therapeutic IFN, which can result in decreased efficacy. It is therefore essential to monitor the development of NAbs by the application of valid bioassay methods. However, to-date, the calculation and reporting of results from such bioassays has been done in various ways leading to non-comparability of results from different laboratories and, in many cases, to scepticism about the accuracy and verity of reported results. Previous Informal WHO Consultations have discussed this matter and made recommendations regarding the neutralization of interferon by antibody (WHO Technical Report Series 687, 1983 and 771, 1987), but without being explicit about how to compute and report results. In addition, two human sera, one containing antibodies against IFN-α and the other against IFN-β, with titres assigned from data analyzed in an international collaborative study, have been established as WHO RR WHO Technical Report Series 858, 1995). The approach recommended at these previous Consultations and by the Standards Committee of the ISICR for the computation and reporting of results is that based on a mathematical model of antibody: antigen interaction developed by Professor Y. Kawade (Kyoto University, Kyoto, Japan). In its current form it allows calculation of neutralizing antibody titre from the dilution of serum that reduces 10 Laboratory Units (LU) per ml of IFN activity to 1 LU per ml, the end-point of most assays (Kawade and Watanabe)7. A refinement to the method of calculation has enabled the titre of antibody that reduces a fixed concentration of IFN (may be more or less than 10 LU per ml) to 1 LU per ml to be calculated from a Kawade formula as Ten-fold Reduction Units (TRU) per ml (Grossberg et al.) 8,9. The use of the Kawade method should make the calculation of neutralizing antibody titre independent of the assay method used, and supportive data to confirm this hypothesis have been accrued.

The Consultative Group recognized that there was a pressing need to harmonize the calculation and reporting of results from not only IFN neutralizing antibody tests but from all other therapeutic protein neutralizing antibody tests. The Group further recognized that a variety of other methods and means of calculating results were currently in use and that the Kawade approach was a valid and useful one for calculating and reporting anti-interferon neutralizing antibody titers, and had led to
the proposal submitted to the ECBS 30 July 2003 by the Standards Committee of the ISICR. The Group, however, felt that it was important to collect more data, using different neutralizing bioassays and means of calculating interferon neutralizing antibodies results by both the Kawade method and any other in current practice, the results of which should be reported together. The Group therefore recommended that:-

- The Kawade approach for the calculation and reporting of results of IFN neutralizing antibody tests is encouraged.
- Investigators are encouraged to use WHO homologous interferon IS to monitor and report the sensitivity of their bioassays, as stated in the ISICR proposal.
- Investigators use both the Kawade and in-house approaches to calculate and express anti-IFN NAb titres and report them side by side, as recommended by the ISICR Standards Committee.
- More data should be collected from either new collaborative studies or manufacturers using alternative neutralizing antibody tests to determine whether the Kawade approach is superior in practice for reducing interlaboratory variability of results and/or confirm its assay independence.

Session 4: Review of on-going projects at NIBSC

Cytokines

Standardization projects aimed at producing WHO IS or RR are currently on-going in the Division of Immunobiology for the following cytokines, all of which are being trialed clinically:

- Thrombopoietin:- definitive fill under evaluation.
- Interleukin-17:- definitive fill of IL-17a under evaluation.
- Interleukin-18:- evaluation of trial fill completed, definitive fill November 2003.
- B-Lymphocyte Stimulator (BLyS):- trial fill under evaluation.

Growth Factors

Standardization projects aimed at producing WHO IS or RR are currently on-going in the Division of Endocrinology for the following growth factors, all of which are being trialed clinically:-

- Keratinocyte Growth Factor (KGF):- candidate standards of natural and modified sequence KGF under evaluation.
- Neurotrophin-3 (NT-3):- candidate standard of NT-3 undergoing stability testing.
- Vascular Endothelial Growth Factor (VEGF):- candidate standard of recombinant VEGF_{165}, the major molecular species occurring naturally, under evaluation.
• Glial cell-derived Neurotrophic Factor (GDNF):- development of in-house bioassay underway, awaiting donation of GDNF for preparation of reference standard.

**Hormones**

• Thyroid stimulating hormone (TSH) for bioassay: - under development.
• Insulin-like Growth Factor-I (IGF-I) for immunoassay: - replacement for existing IRR under development.
• Parathyroid hormone (PTH)₁-₃₄ for bioassay: - bioassay standard (in U) under development to support therapeutic product.

The Consultative Group recognized the importance of the ongoing standardization work being carried out at NIBSC. It recommended notification of this work to WHO ECBS 2003. Priorities for the standardization of new cytokines/growth factors/hormones should be considered by ECBS. The development of reference reagents for pegylated cytokines/growth factors, which are increasingly used clinically, or other modified proteins, e.g., immunotoxins, should be considered.

**Use of WHO international standards and reference reagents of cytokines, growth factors and hormones.**

In summary, use of the IS or RR is typically around 50 laboratories per year, ranges from low (10-20 laboratories per year, e.g., fibroblast growth factor) to high (200-300 laboratories per year (TNF-α, IL-2). One IS (rec hGH) is used at >500 ampoules per year.

The Group noted that:

• all standards have a significant rate of use.
• high use does not necessarily correlate with therapeutic product status (TNF is hardly used as a product).
• the rate of use does not indicate the “importance” of a standard. For example, erythropoietin is only of average use, but nonetheless defines the unit of an extremely important biotech product.
• analysis of geographical distribution data indicates that distribution is concentrated in areas where biotechnology industry is developed.
• however, it is also clear that development of biotechnology in other nations (e.g., Brazil, Argentina, Cuba, Baltic states) is significantly supported by the WHO programme.

The Consultative Group suggested that a database of the use of these reference preparations would be valuable.

**List of Participants**
Dr A. Bristow, Head, Division of Endocrinology, National Institute for Biological Standards and Control (NIBSC), UK;
Dr R. Gaines-Das, Head, Informatics Laboratory, NIBSC, UK;
Dr M. Kohase, Chief, Laboratory of Cytokines, Dept. of Viral Diseases and Vaccine Control, National Institute of Infectious Diseases, Japan;
Dr A. Meager, Division of Immunobiology, NIBSC, UK;
Dr R. Thorpe, Head, Division of Immunobiology, NIBSC, UK;
Ms C. Young, Division of Biotechnology, Korean Food and Drug Administration (KFDA), Republic of South Korea;
Dr K. Zoon, Deputy Director, Center for Cancer Research, USA;
Dr Norman Finter, UK,
Dr A.R. Mire-Sluis, Office of Pharmaceutical Sciences, Center for Drugs Evaluation and Research, National Institutes of Health, USA;
Dr A. Mortimer, Director, Centre for Biologics Research, Biologics and Genetic Therapies, Health Canada, Canada;
Dr Vijay Jethwa, Associate Director, Bioanalytical Quality Control, Biogen, Inc., USA;
Professor Sidney E. Grossberg, Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, USA.

WHO Secretariat

Dr David Wood, Coordinator, QSB, IVB, FCH, WHO;
Dr Ivana Knezevic, Scientist, QSB, IVB, FCH, WHO;
Dr Y. Sohn, ATT, IVB, FCH, WHO.
References


