Standardization of Assays for Human Chorionic Gonadotropin

The unsatisfactory standardization of many immunoassays is fairly well recognized, but the problem is hard to tackle (1). Recently, studies on the performance of immunoassays for testosterone in female serum (2) and for urinary cortisol (1) have revealed that the quality of some widely used assays is totally unsatisfactory. The quality of assays for human chorionic gonadotropin (hCG) has also been shown to be problematic (3, 4), and this was a reason for the IFCC to establish a working group for standardization of assays for this hormone. In addition to being a clinically important analyte, hCG immunoreactivity in biological fluids comprises a reasonable number of well-known variants. hCG was thus considered a suitable model for standardization of glycoprotein hormones in general (5).

It has been argued that it is impossible to standardize assays for heterogeneous antigens (6), and an important aim of the IFCC committee on hCG was to study whether this is the case. Other aims were to introduce a uniform nomenclature, to prepare new standards, and to assign values to these in substance concentrations (mol/L). Standards should preferably be pure, identical to the analyte in serum, and homogeneous, but glycoproteins are inherently heterogeneous because of variation in the carbohydrate moieties. Standards for subunits and degraded forms of hCG occurring in circulation were also needed (5). The working group of the IFCC has achieved most of its goals. Standards for hCG and its most important variants, i.e., its β- and α-subunits (hCGβ and hCGα), the core fragment of hCGβ (hCGβcf), and two partially cleaved or “nicked” forms (hCGn and hCGβn), are now available from WHO as the 1st Research Reagents (1st RRs) (7). The hCG preparation was used by Cole et al. (8) to study how nine widely used immunoassays for hCG are standardized. In addition, the reactivity of these assays with several hCG variants was examined. Their study provides interesting information on causes of between-assay variation and some means to cure these problems.

When serum samples with additions of various partially purified hCG preparations were analyzed with the nine assays, the difference between the highest and lowest result was 1.4- to 1.6-fold. If the results were recalculated on the basis of those obtained for the new 1st RRs for hCG, the maximum variation was reduced to 1.27-fold. The authors draw the conclusion that use of impure assay calibrators in combination with variable recognition of hCG subunits and other variants is an important cause of the variation. Analysis of several commercial hCG preparations revealed that most of them contain degraded forms, subunits, and fragments (8), and earlier studies have shown that rather crude hCG preparations are used as calibrators (4).

The recognition of hCG variants by the studied immunoassays was analyzed by use of purified preparations of hCGβ, hCGn, hCGβcf, and hyperglycosylated hCG. Recognition of these was much more variable than that of “normal” hCG. The immunoreactivities of the variants based on molar concentrations can be estimated on the basis of their molecular weights (7) and the ratio between content in mass units and IU of the 3rd International Standard (IS); i.e., 1 μg = 9.28 IU = 26.7 pmol (5). When analyzed by this method the mean value of hyperglycosylated hCG (900 IU/L = 97 μg/L = 2590 pmol/L) is fairly close to the nominal value (84 μg/L), although one assay underestimated and another one overestimated this variant (52% and 172% of mean, respectively). Thus, hyperglycosylated and normal hCG are recognized fairly equally by most of the assays. This is also the case with hCGn; the mean value (3460 IU/L) corresponds to 373 μg/L, whereas the nominal value is 330 μg/L. The measured mean concentration of hCGβ (3976 IU/L) corresponds to 11 440 pmol/L hCG, which is equal to 269 μg/L hCGβ, whereas the nominal concentration was 230 μg/L. (The values for hCGβ are reported in IU for hCG, according to the 3rd IS, not in those for the 3rd IS for hCGβ, which would give 9.28-fold lower values.) Only one assay detected hCGβcf. The concentration measured, 130 IU/L, corresponds to 0.374 nmol/L hCG, and this corresponds to 3.74 μg/L hCGβcf, i.e., similar to the nominal concentration, 4 μg/L. Thus, with the exception of hCGβcf, no variant is grossly over- or underestimated by any of the assays studied (8). This comparison also demonstrates the advantage of using molar concentrations to express values for various forms of hCG (5).

Cole et al. (8) also studied the effect of differences in sialic acid content, which cause extensive charge heterogeneity in hCG. Charge variants with PI values between 3 and 7 were equally recognized by all assays, showing that variation in sialic acid content does not affect immunoreactivity. This is in agreement with the results of another recent study and show that carbohydrate variation does not usually affect immunoreactivity (9). However, the carbohydrate moieties of hyperglycosylated hCG affected recognition in two assays (10), and one antibody recognizing only a certain form of hyperglycosylated hCG has been described. This antibody, B152, recognizes an epitope comprising the type 2 o-core carbohydrate on Ser132 together with part of the C-terminal peptide of hCGβ. This antibody does not recognize normal hCG containing type 1 o-core carbohydrates (10). The epitope specificities of several antibodies used by assay manufacturers have been carefully characterized (11), but it is not known which antibodies are actually used in the assays studied. It would be interesting to know which epitopes are recognized by the antibodies in assays with aberrant recognition of hyperglycosylated hCG.

What is the significance of the between-assay variation observed? It is obvious that a misleading result may be obtained if different assays are used during monitoring of a patient with suspected ectopic pregnancy or threatening abortion during early pregnancy, when most of the hCG
produced is hyperglycosylated. Specific detection of this form of hCG has been claimed to be of advantage for early detection of pregnancy and pregnancy-related disorders (12, 13). It is reassuring to find that none of the assays failed to detect this form, and thus it is likely that problems will be encountered in pregnancy only when shifting assays during monitoring of the pregnancy.

Between-assay variation is a greater problem for monitoring of cancer patients, who may produce many variants of hCG (4). These patients are followed for long periods of time, which increases the likelihood that different assays will be used. Changes in marker concentrations are particularly important during follow-up of trophoblastic and testicular cancer because therapy of a relapse is often initiated on the basis of increased marker concentrations alone (14). Different recognition of variants is also a more serious problem in cancer than in pregnancy. Trophoblastic tumors produce mainly hCG, but high-risk tumors often produce an excess of hCGβ (15, 16) and aberrantly glycosylated hCG (17, 18). Failure to detect these forms may explain the 58-fold differences among assays observed in an earlier study (4). A recent study of a large number of hCG variants from cancer patients showed that hCG assays using various monoclonal antibodies detect all glycosylated forms (9). Thus it is possible to design hCG assays that detect various forms of hCG equally and to standardize immunoassays for at least this glycoprotein hormone.

Although equal recognition of hCG, hyperglycosylated hCG, and hCGβ is generally desirable, it is also known that separate and specific determination of hCG and hCGβ may facilitate detection of high-risk trophoblastic disease (15, 16). Detection of hCGβ is also of value in the diagnosis of seminomas and nontrophoblastic tumors, many of which produce only this form of hCG immunoreactivity (19, 20). Most commercially available hCG assays detect both hCG and hCGβ, but because the concentrations of hCG are, typically, much higher (up to 16 pmol/L) than those of hCGβ (2 pmol/L), assays measuring these forms together fail to identify moderately increased concentrations of hCGβ (21). Optimum use of hCGβ for this purpose requires specific and sensitive assays, which currently are available in only a few specialized laboratories. Thus there is an unmet need for sensitive and specific assays for hCGβ.

The study of Cole et al. (8) also demonstrates the problems of the 3rd IS for hCG and its subunits, which was one reason to establish the hCG working group (5). Only by expression of concentrations in mol/L can results for various forms of hCG be compared. Because the 1st RR preparations have been assigned values in substance concentrations, it is now possible to calibrate hCG assays in these units, and some groups already do this. The time is ripe for companies to adopt this approach, at least for the subunits. During a transition period, the values could be given both as IU/L according the 3rd IS and in substance concentrations (7). To this end conversion factors need to be established for each method. The results reported by Cole et al. (8) suggest that conversion factors differ among different methods, but systematic studies are needed to reliably establish the conversion factors. The hCG working group is currently planning such a study.

Substance concentrations and conversion factors will not solve all of the problems with between-assay variation. Differences in assay specificity are likely to cause differences in results for samples from some patients, particularly for assays measuring hCG and hCGβ together. It is therefore important that assay manufacturers provide accurate information on assay design and specificity, including epitope specificity of the antibodies used. Several antibodies used in commercial assays have been characterized (11), and it would be valuable if another round of epitope mapping could be organized.

One of the main messages of the study of Cole et al. (8) is that use of the new reference preparation for hCG as a calibrator improves agreement among assays. The available amounts of the very pure 1st RR preparations are not sufficient to be used routinely for this purpose. Very pure recombinant hCG is available and has been used in a commercial assay (22), but it is not known whether it is economically feasible to use it on a large scale. If not, the possibility to prepare sufficiently pure preparations of hCG and hCGβ from urine needs to be explored. Another conclusion that can be drawn is that use of crude urinary hCG preparations in quality-control samples may give misleading results.

Assay standardization requires not only standards but also reference methods (1). The reference method should preferably be a definitive method, e.g., isotope dilution mass spectrometry. To date, however, neither mass spectrometric nor any other reference method is available for hCG or for any other glycoprotein hormone. In the absence of a definitive method, the best method available can be defined as the reference method. The hCG working group has considered evaluating various assays as reference methods. One option would be to define antibodies and conditions that together with the 1st RRs could be applied to various assay formats in any laboratory. Alternatively, a limited number of reference laboratories could maintain the reference methods and prepare sets of calibrated serum samples to be used by manufacturers to check assay calibration (1). This approach has been used to aid manufacturers in the calibration of some steroid hormone assays for which reference methods based on mass spectrometry are available (23).

Comparison of the results in the study of Cole et al. (8) with those in earlier, similar studies shows that the extent of standardization has improved (3, 5), but it is also obvious that further improvement is desirable. The following suggestions are intended to serve as a basis for further discussion on how this can be achieved:

- Use the 1st RRs for hCG and hCGβ to recalibrate existing assays for hCG and hCGβ.
- Establish conversion factors for translation of IU/L to mol/L for existing assays.
• Report results for hCG both in IU/L and mol/L during a transition period.
• Express results for subunits in mol/L.
• Characterize assay specificity with the 1st RR preparations. When necessary, change assay design.
• Report epitope specificities of the antibodies used and, if not known, determine them.
• Identify or prepare preparations free of hCG and hCGβ free of disturbing contaminants to use as calibrators for clinical methods.
• Prepare standards for hyperglycosylated hCG.
• Begin studies to evaluate the feasibility of reference methods and calibrated serum panels.

Some of these recommendations would be easy to realize, whereas others would require extensive and costly changes, at least for some manufacturers. The costs must be weighed against the costs incurred by results causing misinterpretations of clinical conditions. These costs accrue in the clinical wards and may not be visible in the laboratory (1). Cost containment is now a strong driving force in all laboratories, but as laboratorians we need to carefully consider the clinical aspect when optimizing the costs for the laboratory.

References

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