The classification, functions and clinical use of different isoforms of HCG

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HCG is composed of two subunits, HCGα and HCGβ. During early pregnancy, HCG stimulates progesterone production in the corpus luteum, and injection of HCG is widely used to induce ovulation in assisted reproduction treatment (ART). Under experimental conditions, the free subunits have been shown to exert functions other than those of HCG, but the relevance of these remains to be determined. Intact HCG, free subunits and degraded forms of these occur in biological fluids, and determinations of these are important for diagnosis and monitoring of pregnancy, pregnancy-related disorders and several types of cancer. Development of optimal methods for the various forms has been hampered by lack of appropriate standards and expression of the concentrations of the various forms in units that are not comparable. Furthermore, the nomenclature for HCG assays is confusing and in some cases misleading. These problems can now be solved; a uniform nomenclature has been established, and new standards are available for HCG, its subunits HCGα and HCGβ, the partially degraded or nicked forms of HCG and HCGβ, and the beta-core fragment. This review describes the biochemical and biological background for the clinical use of determinations of various forms of HCG. The clinical use of HCG and studies on HCG vaccines are briefly reviewed.

Key words: cancer/Down’s syndrome/HCG isoforms/pregnancy/trophoblastic disease

Introduction

The first assay for HCG described in 1927 was based on the biological activity of HCG partially purified from urine (Ascheim and Zondek, 1927). Various modifications and improvements of this assay were used to diagnose pregnancy until immunoassays gradually replaced bioassays (Wide and Gemzell, 1960; Vaitukaitis et al., 1972). The introduction of monoclonal antibodies facilitated establishment of specific assays for subunits and various degraded forms of HCG, and these have widened the applications of HCG assays for diagnosis of pregnancy, trophoblastic disease and non-trophoblastic cancers (Stenman et al., 2004). Thanks to the high concentrations of HCG in urine during pregnancy, urine has been used for the preparation of HCG for clinical use, and partially purified urinary HCG plays an important role in assisted reproduction treatment (ART). This use prompted establishment of standards for HCG, which were assigned values based on bioactivity. All the international standards (ISs) for HCG have been calibrated by bioassay (Storring et al., 1980). Although not ideally suited, these standards have also been used for standardization of immunoassays (Stenman et al., 1993). Furthermore, the nomenclature used to describe HCG assays is confusing and often misleading. To solve these problems, the International Federation of Clinical Chemistry (IFCC) established a working group in 1995 with the aim of improving standardization of HCG determinations; the task included establishment of a uniform nomenclature and preparation of new standards. At that time, the clinically important variants were HCG and its subunits HCGα and HCGβ, the partially degraded or nicked forms of HCG (HCGn) and HCGβ (HCGβn), and the beta-core fragment (HCGβcf) (Stenman et al., 1993). This nomenclature has been adopted by IFCC and the aim is to get it accepted by the scientific community. In addition to these variants, recently described glycosylation variants are of potential clinical utility. This review describes the classification and functions of various forms of HCG and the clinical use of HCG assays for diagnosis of pregnancy, pregnancy-related disorders and gynaecological cancers. Recent developments in the therapeutic use of HCG and studies on HCG vaccines are also described. The aim has been to critically discuss how earlier findings can be interpreted in the light of recent advances in biology and assay technology.

Biochemistry of HCG

HCG is a member of the glycoprotein hormone (GPH) family, which also comprises LH, FSH and TSH. All GPHs are heterodimers consisting of an α-subunit (GPHα) and a β-subunit. The α-subunit, which contains 92 amino acids, is common to all GPHs. The β-subunits confer biological activity and display various degrees of homology, which between HCG and LH is ~80%. LHβ contains
121 amino acids whereas HCGβ contains 145 amino acids, the difference being due to a 24-amino-acid extension, the so-called C-terminal peptide (CTP) (Pierce and Parsons, 1981).

One-third of the mass of HCG is made up by eight carbohydrate moieties, of which six are attached to HCGβ and two to HCGα. The N-linked carbohydrate chains on HCGα are attached to Asn52 and Asn78 and those on HCGβ to Asn13 and Asn30. Four O-linked oligosaccharides are attached to Ser121, Ser127, Ser132 and Ser 138 on the CTP of HCGβ (Kessler et al., 1979a,b; Elliott et al., 1997). Owing to variation in the content of terminal sialic acid, HCG displays extensive charge heterogeneity with isoelectric point (pI) values ranging from 3 to 7. HCGβ is more acidic (pI range 3–5) than HCGα (pI range 5–8) (Graesslin et al., 1973; Birken et al., 2003). In pregnancy, the N-linked carbohydrates on HCGα are mainly monoantennary and biantennary and those on HCGβ are biantennary and to a lesser extent triantennary. The carbohydrates on the CTP are mainly monoantennary type 1 o-core oligosaccharides. In HCG produced by cancerous tissues, most of the N-linked carbohydrates are complex containing more triantennary moieties on HCGβ (Mizuochi et al., 1983) and biantennary on HCGα, whereas type 2 o-core oligosaccharides occur on CTP (Elliott et al., 1997; Birken, 2005). This so-called hyperglycosylated HCG (HCGh) is also a major form in early pregnancy (Kovallevskaya et al., 2002a). It is produced by cytotrophoblasts, which dominate in the early placenta, whereas syncytiotrophoblasts, which are the main trophoblasts later in pregnancy, produce ‘normally glycosylated’ HCG (Kovallevskaya et al., 2002b). In addition to these differences, HCG produced by trophoblastic cancers occasionally displays reduced content of sialic acid (Nishimura et al., 1981; Imamura et al., 1987), but variants with unusually low pI values indicating increased content of sialic acid have also been described (Yazaki et al., 1987). Thus, various forms of aberrant glycosylation are common in tumour-derived HCG (Kobata and Takeuchi, 1999).

Because of heterogeneity of the CHO moieties, the molecular weight (MW) displays a spectrum of values. The average MW of HCG determined by MALDI-TOF mass spectrometry is 37 500, that of HCGα is 14 000 and that of HCGβ is 23 500 (Birken et al., 2003). The calculated mass of the peptide moiety of HCGα is 10 206 and that of the glycosylated subunit containing two biantennary N-linked sialylated oligosaccharides is 14 165. The MW of the HCGβ peptide is 15 532 and that containing two N-linked biantennary CHO chains and four type 1 o-core carbohydrates is 24 316. Thus, the calculated MW of a ‘typical’ HCG molecule would be 38 931. The difference between the calculated and measured average MW indicates that the CHO chains are on average smaller than those used to calculate the theoretical MW. The MW of HCG produced by trophoblastic cancer is higher than that of pregnancy HCG (Mann and Karl, 1983), which is explained by larger carbohydrate chains (Elliott et al., 1997; Birken, 2005).

Part of the heterodimeric HCG in urine is nicked (HCGn), i.e. the peptide chain is cleaved at various positions between amino acids 44 and 48. This nicked HCG may also occur in the serum of cancer patients (Cole et al., 1991; Jacoby et al., 2000). Part of the HCGβ isolated from urine is also in the HCGn form. HCG lacking the CTP may occur in urine of some cancer patients (Cole et al., 1982).

Most of the HCG immunoreactivity in urine from pregnant women (Mathies and Diczfalussy, 1971) and cancer patients (Papapetrou et al., 1980; Wehmann and Nisula, 1980) consists of the beta-core fragment, HCGβcf (Stenman et al., 1993), which has been shown to comprise amino acids 6–40 and 55–92 linked by disulphide bridges. The carbohydrate moieties on Asn 13 and 30 are smaller than in those on intact HCG (Birken et al., 1988).

Genes

Six non-allelic genes clustered on chromosome 19q13.3 encode HCGβ, and a seventh gene encodes LHβ (Fiddes and Goodman, 1980). Genes β1 and β2 are thought to be pseudogenes that are not expressed; β4 encodes LH whereas β7 and β9 are alleles of β6 and β3, respectively. Type I genes (β6/β7) encode a protein with alanine at position 117 whereas HCGβ encoded by type II genes (β3/β9, β5 and β8) contains aspartic acid at this position. This heterogeneity is not known to affect function or immunoreactivity. Type I genes are mainly expressed in benign non-trophoblastic tissues whereas type II genes are expressed by trophoblastic and malignant tissues (Bellet et al., 1997). A single gene on chromosome 12q21.1–23 encodes GPHα (Fiddes and Goodman, 1981).

Function

HCG mediates its action through the LH/HCG receptor, and its major function is to maintain the progesterone production of corpus luteum during early pregnancy. Various other tissues also express the LH/HCG receptor, and its presence in the vasculature of the uterus may indicate that HCG exerts a physiologically important function in this tissue. The receptor is also expressed in a large number of tissues other than the ovary, and thus HCG and LH may have hitherto unknown functions. Known and putative functions of HCG have recently been extensively reviewed (Filiocori et al., 2005).

HCGh is produced by cytotothrophblast during early pregnancy (Kovallevskaya et al., 2002b). Because these cells display invasive properties (Red-Horse et al., 2004), HCGh has also been called invasive trophoblast antigen (ITA) (Cole et al., 1999). However, there is so far no evidence for an invasive function and no receptor other than the LH/HCG receptor has been identified.

HCGβ lacks HCG activity, but several lines of study indicate that it exerts growth-promoting activity. It enhances growth of bladder cancer cells, and antibodies to HCGβ inhibit this effect (Gillott et al., 1996; Butler et al., 2003). In rat breast cancer cells, HCGβ has been shown to induce apoptosis (Srividavata et al., 1997) and inhibition of HCGβ expression with antisense messenger RNA suppresses cell proliferation and induces apoptosis in chorionicarcinoma cells (Hamada et al., 2005). However, a mechanism mediating this activity has not been found, but based on structural similarity, it has been speculated that HCGβ interferes with the growth-inhibiting effect of transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF)-B and nerve growth factor (Butler and Iles, 2004).

HCGα (GPHα) also does not exert HCG activity, but it has been shown to stimulate prolactin production in decidual cells (Blithe et al., 1991; Moy et al., 1996). Furthermore, endometrial cells induce dissociation of HCG into subunits, and together with progesterone, the released HCGα may mediate decidualization of these cells (Nemansky et al., 1998).

Metabolism

The clearance of HCG from circulation has been studied both after injection of purified HCG and after pregnancy. The half-life of
Injected HCG is biphasic; the rapid phase has a half-life of 5–6 h whereas that of the slower phase is 24–33 h (Rizkallah et al., 1969; Wehmann and Nisula, 1981). Similar clearance rates have been observed after an abortion and term pregnancy, but the clearance is best described by a triphasic model with median half-lives of 3.6, 18 and 53 h (Korhonen et al., 1997). The half-life of purified HCGβ injected into humans is 0.7 and 10 h, which is shorter than that of HCG (Wehmann and Nisula, 1979). However, after term pregnancy or an abortion, HCGβ actually disappears more slowly than HCG with half-lives of 1, 23 and 194 h. Thus, the proportion of HCGβ of total HCG immunoreactivity increases from 0.8% at term to 27% after 3 weeks. The half-life of HCGα is shorter than that of HCGβ, and after term pregnancy half-lives of 0.6, 6 and 22 h have been observed (Korhonen et al., 1997). These half-lives are longer than those observed after injection of purified HCGα, i.e. 0.1–0.22 and 1.2–1.3 h (Wehmann and Nisula, 1979; Blithe and Nisula, 1987). The discrepancy between half-lives determined for injected and naturally occurring subunits may indicate that the purified forms have been partially denatured during purification, and they are therefore metabolized more rapidly. It is also possible that the slower metabolism of endogenous free subunits is explained by differences in glycosylation.

Most of the HCG in circulation is metabolized by the liver, whereas about 20% is excreted by the kidneys (Nisula et al., 1989). During excretion, a major part of HCG is degraded to subunits, nicked forms and especially HCGβcf (Wehmann and Nisula, 1980; Nisula et al., 1989). The proportion of HCGβcf in urine is low in early pregnancy and starts to exceed that of HCG at ~5 weeks of pregnancy (Figure 1). In the second trimester, about 80% of the HCG immunoreactivity in urine consists of HCGβcf (Norman et al., 1987). During pregnancy, HCGβcf can be detected in plasma (Nisula et al., 1989; de Medeiros et al., 1992b), but the concentrations are only ~0.01% of those of HCG (Alfthan and Stenman, 1990). After injection of urinary HCG, HCGβ or recombinant HCG (rHCG), HCGβcf is detected in urine (Nisula et al., 1989), but peak concentrations occur ~6 h after the HCG peak in urine (Norman et al., 2000). HCGβcf can also be detected in the pituitary (Hoermann et al., 1995) and in follicular fluid and trophoblast culture fluid (de Medeiros et al., 1992a), and some HCGβcf is present in the placenta (Udagawa et al., 1998). Thus, some of the HCGβcf in urine can be derived from metabolism in these tissues, but studies on the metabolic clearance rate of HCGβcf show that >99% is formed in the kidneys during renal excretion (Wehmann et al., 1989).

### Standards for HCG, its subunits and fragments

The World Health Organization (WHO) has issued several sets of standards for HCG, which have been calibrated in IU against the preceding set by bioassay. The presently used third and the identical fourth ISs were prepared in 1972. They have been available from NIH as CR119 and were initially issued by WHO as the 1st International Research Preparations (1st IRP) (Canfield and Ross, 1976) and adopted as the third IS WHO standards in 1980 (Storring et al., 1980). They comprise standards for HCG (75/537), HCGβ (75/551) and HCGα (75/569). The HCG standard was calibrated by bioassay against the 2nd standard, and 1 μg of HCG in the third IS corresponds to ~9.3 IU. Because the free subunits lack HCG activity, they were assigned values based on mass with 1 μg corresponding to 1 IU (Storring et al., 1980). Thus, the units are not comparable with those of HCG. Table I summarizes the relationship between the units for HCG and its subunits. When HCGβ is measured by assays detecting HCG and HCGβ together, the results are erroneously based on the IU for HCG.

The preparations in the third IS have been found to be contaminated with partially degraded variants of HCG, which is a problem when they are used for standardization of immunoassays. To solve these problems, the IFCC founded a working group with the aim of improving standardization of immunoassays for HCG and related molecules (Stenman et al., 1993). As a result of this project, new standards for HCG and clinically important HCG-related molecules have been prepared and approved by the WHO as reference reagents for immunoassay (Table II). Purer preparations than those in third IS were produced by utilizing modern chromatographic techniques. However, WHO recommends that the third (and fourth) IS still should be used for calibration of immunoassays. The purification and value assignment of the reference reagents have been described (Birkén et al., 2003; Bristow et al., 2005).

Because immunoassays reflect molar concentrations of protein rather than bioactivity, the use of substance concentrations, i.e. mol/l is the most appropriate way of expressing the concentrations. This is especially important when two (or several) analytes of different MW are detected together or when their concentrations are compared (Stenman et al., 1993). This is a major reason why the reference reagents for HCG and related substances have been assigned values in molar concentrations based on amino acid analysis (Birkén et al., 2003; Bristow et al., 2005). Furthermore, the carbohydrate composition has little effect on immunoreactivity, but it strongly affects bioactivity. Because the new reference

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**Table I. Comparison of various units for the third international standards for HCG and its subunits**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Molecular weight</th>
<th>μg/IU</th>
<th>pmol/IU</th>
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<tbody>
<tr>
<td>HCG</td>
<td>37 500</td>
<td>0.11</td>
<td>2.9</td>
</tr>
<tr>
<td>HCGβ</td>
<td>23 500</td>
<td>1</td>
<td>42.5</td>
</tr>
<tr>
<td>HCGα</td>
<td>14 000</td>
<td>1</td>
<td>71.4</td>
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preparations are more pure than the third IS, the bioactivity of the HCG preparation, about 11 000–16 000 IU/mg, is 20–70% higher than that of the third IS (Birken et al., 2003). Thus, it is obvious that value assignment on the basis of bioactivity would have resulted in a substantial change in immunoassay calibration. The higher bioactivity is explained by absence of biologically inactive HGN and HCGβcf in the new HCG preparation. When the new reference reagents are used to calibrate immunoassays for HCG, HCGβ, HCGα and HCGβcf, the concentrations should be expressed in pmol/l.

The reference reagents comprise six forms of HCG which were considered clinically important when the project was initiated. Some of them, e.g. HCGn, are valuable for the characterization of antibody specificity. Antibodies that do not recognize HCGn will miss this variant, which occurs especially in urine but also in the serum of some cancer patients (Cole et al., 1991; Hoermann et al., 1994). HCGβn is likewise mainly of importance for the characterization of the epitope specificity of antibodies (Berger et al., 2002). HCGβcf is of potential value as a marker for cancer (Papapetrou et al., 1980) and pre-eclampsia (Bahado-Singh et al., 1998), but assays for this variant have not become clinically accepted. As HCGβcf represents a major part of the HCG immunoreactivity in urine of pregnant women, the standard is important for the characterization of pregnancy tests and other assays for HCG immunoreactivity in urine.

When the reference reagents were prepared, the potential utility of HCGh was not recognized and a standard for this variant was not prepared. If HCGh assays become clinically accepted, a standard will be desirable. However, identification of a suitable source is problematic; although HCGh occurs in urine during early pregnancy (Kovalevskaya et al., 1999), it is not yet known whether this HCGh is comparable with that occurring in patients with trophoblastic and other types of cancer. It is well known that the carbohydrate composition of HCG produced by tumours may vary considerably, and HCGh is therefore not a well-defined entity (Birken, 2005). Recently, HCGh has been named ITA (Cole et al., 1999), but so far there is no evidence for an invasive function and a separate name for this form is mainly motivated by commercial interests. This does not justify the introduction of a new name in the scientific literature.

rHCG is also available as a laboratory product and is a potential calibrator for immunoassay purposes. Part of the variation between different immunoassays is caused by the use of impure calibrators, and improved agreement may be obtained by replacing the calibrators in commercial assays by pure HCG preparations (Cole et al., 2004). However, this is only one cause of between-method discrepancies, careful selection of antibodies and assay design being equally important (Stenman et al., 1993; Berger et al., 2002). rHCG would not be useful for calibration of assays based on antibody B152, which recognizes HCGh.

**Therapeutic use of HCG**

The midcycle LH surge is essential for normal oocyte maturation and ovulation. In ART, administration of partially purified urinary HCG preparations has been used for decades as a surrogate for LH to achieve final oocyte maturation and ovulation in controlled ovarian hyperstimulation (COH) protocols. This facilitates correct timing of oocyte retrieval in connection with IVF/ICSI treatments. Urinary HCG has been the drug of choice, but there are now other options, i.e. recombinant LH (rLH) and HCG (rHCG). rLH has been available for use in clinical trials for several years. A single dose of 15 000–30 000 IU of rLH gives the highest efficacy to safety ratio. Such a dose is comparable with 5000 IU of urinary HCG, and it effectively induces final follicular maturation and early luteinization in IVF embryo transfer patients (The European recombinant LH study group, 2001). However, rLH has not been shown to be superior to HCG in clinical practice. Moreover, it has recently been suggested that replacement of urinary HCG by rLH in agonist cycles results in a significantly lower pregnancy rate (Aboulishar and Al-Imany, 2005).

For many years, the most widely used form of COH in IVF/ICSI protocols has been to use GnRH agonists to desensitize the pituitary and suppress gonadotrophin secretion, followed by ovarian stimulation with FSH and/or HMG. Presently, GnRH antagonists are an alternative in preventing premature LH surges in COH. Because the pituitary remains responsive to GnRH agonists, administration of a bolus of GnRH agonist induces an endogenous LH surge (Griesinger et al., 2006). This form of treatment has been suggested to prevent the ovarian hyperstimulation syndrome (OHSS) (Orvieto, 2005). In clinical studies, ovulation induction with the GnRH agonist buserelin resulted in significantly more mature oocytes, but significantly lower implantation and clinical pregnancy rates were obtained than those by conventional ovulation induction with urinary HCG (Humaidan et al., 2005). Moreover, the rate of early pregnancy loss was higher, probably due to luteal phase deficiency. This has been confirmed in other studies, and a lower probability of ongoing pregnancy was achieved with the GnRH agonist triptorelin than with urinary HCG (Kolibianakis et al., 2005). At present, HCG appears to be the most reliable way.
to trigger final oocyte maturation both in antagonist and in agonist cycles (Griesinger et al., 2006).

It seems that similar characteristics and dynamics of luteal phase estradiol (E₂) and progesterone are obtained after ovarian stimulation for IVF using GnRH agonists or antagonists (Friedler et al., 2006), and thus luteal support is needed in both protocols. Luteal phase support after ART results in an increased pregnancy rate compared with placebo or no treatment. When HCG is compared with progesterone treatment, there is no significant difference in pregnancy rates but HCG is associated with a greater risk of OHSS (Daya and Gunby, 2004).

Urinary HCG can also be used for other clinical applications. Thus low-dose HCG has been used alone to complete controlled ovarian stimulation (Filicori et al., 2005). When used in the late stage of ovarian stimulation (after the follicles were ≥12 mm), this reduces FSH/HMG consumption while fertilization outcome is comparable. Furthermore, HCG use is associated with a reduced number of small pre-ovulatory follicles, which could reduce the risk of OHSS (Filicori et al., 2005). Even more interesting is the effect of HCG on uterine receptivity. In a recent study, administration of HCG to oocyte recipients was shown to increase endometrial thickness on the day of embryo transfer and to improve the implantation rates (Tesarik et al., 2003). This suggests that HCG might affect endometrial function independently of ovarian function by stimulating endometrial growth and maturation and by enhancing endometrial angiogenesis, thereby extending the implantation window (Filicori et al., 2005).

rHCG

Recently, HCG produced by recombinant techniques in Chinese hamster ovary cells has become commercially available. In ART, a dose of 250 μg of rHCG has been found to be equivalent (Chang et al., 2001) or at least as effective as 10,000 IU of urinary HCG in inducing final stages of oocyte maturation. Furthermore, the use of rHCG was associated with significantly better patient tolerance (Abdelmassih et al., 2005). The content of HCG in urinary preparations for therapeutic use is expressed in IU based on bioactivity whereas that of rHCG is expressed in mass units (Gervais et al., 2003), which complicates comparison of dosage. In animal and clinical studies, 250 μg of rHCG has been found to have the same biological activity as 5000 IU of urinary HCG (Gervais et al., 2003; Al-Inany et al., 2005). This would translate into a specific activity of 20,000 IU/mg, which is higher than the potency of 15,000 IU/mg of the most pure urinary HCG preparations presently available (Birken et al., 2003). However, the content of rHCG is based on the mass of the peptide moiety only (Gervais et al., 2003), and thus 250 μg corresponds to ~360 μg of glycosylated HCG and a specific activity of 13 900 IU/mg. This is comparable with that of highly purified urinary HCG but, according to the earlier mentioned studies, the activity may be even higher. These calculations are based on the typical carbohydrate structure of urinary HCG, and the carbohydrates of rHCG are only slightly different; the N-linked carbohydrates are of the same type as those in urinary HCG, but part of the O-linked carbohydrate chains on the CTP is different (Gervais et al., 2003). So far, this has not been reported to cause any adverse effects indicating that the carbohydrate moieties are not immunogenic. The half-life of rHCG in circulation is similar to that of urinary HCG (Gervais et al., 2003).

HCG isoforms: classification, functions, clinical use

Immunological determination of HCG

Assay nomenclature

Because of the extensive homology between HCG and LH, the first radioimmunoassays (RIAs) for HCG based on polyclonal antiserum also measured LH (Odell et al., 1967). A quite specific rabbit antiserum (SB6) to HCG was produced in 1972 by immunization with HCGβ, and an RIA based on this antisera detected HCG at concentrations down to 5 IU/L (Vaitukaitis et al., 1972). This assay has been widely employed (Hussa, 1987), which probably explains why the expressions ‘β-HCG assay’ or ‘HCG-beta assay’ have become commonly used. Initially, these names implied that the assays did not cross-react with LH, but presently, they are mostly used for assays measuring HCG and HCGβ together. IFCC recommends that assays should be exactly defined according to what they measure, e.g. HCG and HCGβ separately or together (Stenman et al., 1993). Thus, an HCGβ assay should detect HCGβ but not HCG.

Effect of antibody epitopes on assay specificity

Although specific assays for HCG and its various forms can be established with polyclonal antisera, virtually all presently used assays utilize monoclonal antibodies or a combination of a monoclonal antibody and a polyclonal antisem. Monoclonal antibodies with known epitope specificity facilitate design of assays specific for each form of HCG (Bidart et al., 1985; Ehrlich et al., 1985; Norman et al., 1985; Schwartz et al., 1986; Alffman et al., 1992a). The antigenic regions on HCG have been extensively defined; five epitopes can be discerned (Berger et al., 1994). Two epitopes are specific for each free subunit, α₇ and β₉, positioned on the CTP, are completely specific for HCG and HCGβ, and antibodies recognizing these epitopes are therefore used in many commercial assays. Four epitopes (C₁–C₄) are specific for heterodimeric HCG. Two of these conformation-dependent epitopes, C₁ and C₂, are lost in HCGn (Hoermann et al., 1994). Two epitopes are specific for each free subunit, α₇ and α₁, for HCGα and β₉ and β₁, for HCGβ (Berger et al., 1996; Berger et al., 2002).

The epitopes of 28 antibodies from various manufacturers and research groups have been determined in a collaborative study. On the basis of this information, the specificity of an assay in which these antibodies are used can be deduced (Berger et al., 2002). Exact information on assay specificity is obtained by analysing the reference reagents with the final assay (Birken et al., 2003).

Most epitopes are not dependent on variation in carbohydrate composition (Schwartz et al., 1991; Lottersberger et al., 2003), but two monoclonal antibodies have been shown to recognize certain carbohydrate variants on HCG. Antibodies B152 and CTP 104, which were prepared against aberrantly glycosylated HCG isolated from the urine of a choriocarcinoma patient, recognize carbohydrate epitopes (Kovaleskaya et al., 1999). That of B152 comprises the biantennary core 2 α-glycan on Ser138 and adjacent peptide structures whereas CTP 104 reacts with a sialylated glycan on Ser138 (Birken, 2005). A number of recent studies suggest that assay of HCGβ is clinically useful in certain clinical conditions. The expression ‘hyperglycosylated’ was initially used to denote HCG containing complex carbohydrates (Elliott et al., 1997), but it is presently mainly used to denote HCG determined by assays
using antibody B152. This nomenclature is an oversimplification of the actual complexity of the carbohydrate heterogeneity of HCG (Birken, 2005).

Design of assays for various clinical purposes

Serum samples are preferred for quantitative HCG determinations whereas urine samples are mainly used for pregnancy tests. Because both HCG and HCGβ may occur in serum, most serum assays are designed to measure these together. Presently, virtually all commercial assays are based on the sandwich principle (Cole et al., 1997), and some assays utilize an antibody to CTP in combination with another antibody to HCGβ. Such assays show no cross-reactivity with LH, but because CTP antibodies tend to have only moderate affinity (Berger et al., 2002), these assays are not very sensitive. Furthermore, HCG lacking CTP, which may occur in cancer (Cole et al., 1982), is not detected. Some assays detect HCG and HCGβ fairly equally, but there are still considerable differences in this respect (Cole et al., 2004). Assays specific for HCG can be designed by using one antibody to HCGβ (usually to capture HCG) together with an antibody to HCGα as a tracer. Some of the most sensitive assays for HCG are based on this principle (Pettersson et al., 1983; Alfthan et al., 1992a), and they are also easy to standardize (Stenman et al., 1993).

Assays detecting HCG, HCGβ and HCGβcf together have advantages for the measurement of HCG immunoreactivity in urine (Cole and Butler, 2002; McChesney et al., 2005). Immunoassays based on the binding inhibition principle, i.e. classical RIAs, mostly recognize all these forms, but few commercial HCG assays based on the sandwich principle do so (Cole et al., 2001; Cole et al., 2004). Urine samples are mainly useful for the identification of false-positive results in serum samples (Stenman et al., 2004), but for instance in the UK, they are also used to detect a relapse after treatment of trophoblastic tumours in outpatients (Mitchell, 1999).

Specific and sensitive assays for HCGβ can be developed by using monoclonal antibodies (Ozturk et al., 1987; Alfthan et al., 1988; Marcillac et al., 1992; de Medeiros et al., 1992a). Most commercially available HCGβ assays are intended for maternal screening of Down’s syndrome, and being optimized for the high serum concentrations occurring in pregnancy, they are not well suited for the determination of the low levels of HCGβ that typically occur in the serum of cancer patients (Stenman et al., 2004).

Many non-trophoblastic tumours produce HCGβ, most of which is degraded to HCGβcf when excreted into urine (Alfthan et al., 1992b). Several assays for HCGβcf in urine have been described and shown to be useful especially for the detection of gynaecological cancers (Cole et al., 1988; O’Connor et al., 1988; Alfthan et al., 1992a; de Medeiros et al., 1992a; Neven et al., 1993). Some assays measure several degraded forms of HCGβ (de Medeiros et al., 1992a), which collectively have been called urinary gonadotrophin fragments (Cole et al., 1988) and urinary gonadotrophin peptides (Schwartz et al., 1996). The use of these assays for monitoring of cancer is hampered by large day-to-day variation in the urine concentrations of HCGβcf, which is not eliminated by normalization against urinary creatinine (Ngan et al., 1995). Because of these problems, commercial assays for HCGβcf are presently not widely available. In patients with non-trophoblastic cancers, the concentrations of HCGβcf in urine reflect those of HCGβ in plasma (Alfthan et al., 1992b) and, when measured by a highly sensitive assay, HCGβ in serum is the more accurate marker (Alfthan et al., 1992b).

Pregnancy tests

The most common use of HCG determinations is the detection of pregnancy with a semi-quantitative pregnancy test, which mostly is performed on a urine sample, but serum, plasma or whole blood can also be used with some tests. The pregnancy test is considered one of the most useful and reliable laboratory tests available (Chard, 1992). The first immunological pregnancy test based on haemagglutination inhibition had a detection limit of 500 IU/l and took 1.5 h to perform (Wide and Gemzell, 1960). The agglutination assays were gradually replaced by more sensitive and rapid enzyme immunoassays, which facilitated detection of HCG at concentrations down to 25 IU/l in 5–10 min (Wide, 2005). Presently, most pregnancy tests are based on immunochromatography and have a claimed sensitivity of 25–50 IU/l. However, some are actually more sensitive, detecting HCG at concentrations <10 IU/l while the detection limit of other pregnancy tests is in the range 100–200 IU/l (Cole et al., 2005).

The optimal sensitivity of a pregnancy test is debated; the more sensitive a test is, the earlier it detects a pregnancy, but because HCG may occur in serum and urine at concentrations up to 10–15 IU/l in non-pregnant women, a detection limit around 25 IU/l is considered optimal (Chard, 1992; Stenman and Alfthan, 2003). Determination of the sensitivity of a pregnancy test depends on the standards used. Much of the HCG immunoreactivity in urine consists of HCGβcf, but during the 5–7 first weeks of pregnancy, i.e. when pregnancy tests are used, HCG is the dominating form (Figure 1). Free subunits are also of minor importance, but failure to recognize HCGh and degraded forms of HCG, e.g. HCGn, is a potential problem (Butler et al., 2001). However, nicking of HCG occurs mainly during storage of urine (Birken et al., 2001), which is not a problem in pregnancy testing. Some pregnancy tests have been shown to underestimate HCG (Butler et al., 2001), but these studies were performed on spiked samples and it remains to be determined whether this is a problem in fresh urine samples. There is considerable variation in the performance of pregnancy tests sold to the general public (Cole et al., 2005), but little information is available on the performance of presently used pregnancy tests for professional use.

Reference values for serum

Serum from men and non-pregnant women contain low levels of HCG and HCGβ that can be detected by sensitive assays. Pituitary GPHα is produced in excess of the β-subunits, relatively high levels occur normally, and they increase after the menopause. However, GPHα is not increased in patients with non-trophoblastic cancers that produce HCGβ (Braunstein et al., 1979). Assay of GPHα has been reported to be useful for the diagnosis of testicular cancer (Mann and Karl, 1983), but this application is unusual and generally applicable reference values have not been established. Suppression of gonadotrophin secretion with GnRH analogues causes a continuous increase in GPHα when the pituitary gonadotrophin production decreases (Unkila-Kallio et al., 2000). Thus, the regulation of GPHα expression in the pituitary is different from that of the GPHβ subunits.
The HCG levels expressed in IU/l are about 3–10% of those of LH, and they increase after menopause in women and after age 60 in men (Alfthan et al., 1992a). The secretion of pituitary HCG is regulated by GnRH, and elevated levels in post-menopausal women are suppressed by estrogen treatment (Stenman et al., 1987). The serum concentrations fluctuate in a pattern similar to that of LH (Odell and Griffin, 1987). Taken together, these results indicate that most HCG in normal serum is derived from the pituitary (Stenman et al., 1987). Low-level expression of the genes for both HCG subunits occurs in the testis, breast, prostate and skeletal muscle (Bellet et al., 1997), but it is not known whether these tissues contribute to the levels of HCG in circulation.

The concentrations of HCGβ in the serum of healthy men and non-pregnant women are low and measurable in only some of the samples even with the most sensitive assays available. The concentrations do not increase with age (Alfthan et al., 1992a). The genes for HCGβ are expressed at very low levels in many tissues without concomitant expression of HCGα, e.g. bladder, adrenal, colon, thyroid and uterus, but the source of the ‘normal’ levels of HCGβ in serum is not known (Bellet et al., 1997).

Table III summarizes the reference values for HCG and HCGβ in serum and urine. To facilitate comparison, we expressed the concentrations in substance concentrations (pmol/l) and for HCG also in IU/l. One IU corresponds to 0.11 μg and 2.9 pmol of HCG, and thus the values in pmol/l are roughly 3-fold those in IU/l. The conversion factors for HCGβ are quite different, with 1 IU corresponding to 1 μg and 42.5 pmol. The concentrations of HCGβ are actually seldom expressed in IU/l based on its own standard (the third IS) but rather in pmol/l (Stenman et al., 1993).

The upper reference limits for HCG based on the 97.5 percentile are 3 and 5.4 IU/l in fertile and post-menopausal women, respectively, and those for men below and above 60 years of age are 0.7–2.1 IU/l, respectively (Table III). However, occasional values up to 14 IU/l were observed in post-menopausal women (Snyder et al., 1992a). Other than the pregnancy test itself, hormone measurements are seldom used for monitoring of pregnancy or cancer, but the reference limits are of value when urine samples are used to confirm false-positive results with serum assays.

Table III. Upper reference limits for serum and urine concentrations of HCG, HCGββ and HCGββcf in non-pregnant women and in men

<table>
<thead>
<tr>
<th></th>
<th>Women 97.5 percentile</th>
<th>Men 97.5 percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;50 years</td>
<td>≥50 years</td>
</tr>
<tr>
<td>Serum</td>
<td>pmol/l</td>
<td>IU/l</td>
</tr>
<tr>
<td>HCG</td>
<td>8.6</td>
<td>3.0</td>
</tr>
<tr>
<td>HCGβ</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Total HCG</td>
<td>9.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Urine</td>
<td>pmol/l</td>
<td>IU/l</td>
</tr>
<tr>
<td>HCG</td>
<td>8.8</td>
<td>3.1</td>
</tr>
<tr>
<td>HCGβ</td>
<td>1.7</td>
<td>4.3</td>
</tr>
<tr>
<td>HCGββcf</td>
<td>8.1</td>
<td>9.5</td>
</tr>
<tr>
<td>Total HCG</td>
<td>13.6</td>
<td>20.4</td>
</tr>
</tbody>
</table>

Figure 2. Serum concentrations of HCG during normal pregnancy. Note that the concentrations are expressed in pmol/l. The values can be converted to IU/l by dividing them by 2.9.

Reference values for pregnancy

The serum concentrations of HCG start increasing 7–10 days after the LH peak or 4–7 days after implantation (Lenon et al., 1982). During early pregnancy, the HCG concentrations increase exponentially doubling on average every 1.5–2 days, but the rate of increase varies individually and maximum concentrations ranging from 20 000 to 100 000 IU/l are reached at 7–10 weeks of pregnancy. After this, the levels decrease levelling out at 13–15 weeks and increase moderately again until weeks 30–33, after which there is a moderate decrease towards term (Alfthan et al., 1988) (Figures 1–3).
The serum concentrations of HCG correlate strongly with those of HCG, but the proportion of HCGβ changes during pregnancy. In early pregnancy, it may reach 4% but it drops rapidly to <1% (Figure 4). Accurate reference ranges for HCG and HCGβ during pregnancy are especially important for the diagnosis of Down’s syndrome, and owing to the rapid changes during pregnancy, the values are expressed as multiples of the median for each week (or even day) of pregnancy (discussed below). Because of between-method differences in calibration, it is essential that the reference values be established separately for each assay.

The HCG immunoreactivity in pregnancy urine is more heterogeneous than that in serum, and therefore the results are strongly dependent on the specificity of the assay used. When measured by specific assays, the HCG concentrations in urine correlate strongly with those in serum. In early pregnancy, the average urine concentrations are 50–70% of those in serum, but after the fifth week, the urine-to-serum ratio of HCG decreases and HCGβcf becomes the dominant form in urine (Figure 1) (Norman et al., 1987). In most studies, HCGβ has been found to be a minor component in urine (Norman et al., 1987). The proportions of the various immunoreactive forms vary considerably from day to day, whereas the sum of the various forms shows less variation indicating that loss of HCG is reflected by increased levels of degraded forms such as HCGβ and HCGβcf (McChesney et al., 2005) (Figure 1). The large day-to-day and within-day variation limits the clinical utility of quantitative urine assays. The variation in urinary HCG concentration can be reduced by normalizing against urine density or urine creatinine concentration. However, this eliminates only part of the variation and serum assays are therefore better suited for quantitation. Because quantitative urine assays are not used for monitoring of pregnancy, appropriate reference values have not been established.

Use of HCG assays in pregnancy

Monitoring of pregnancy
Quantitative determinations of HCG are used to predict complications especially in early pregnancy, e.g. pregnancy loss and ectopic pregnancy. About 20–30% of all pregnancies end in an early pregnancy loss, which often takes place before the pregnancy is clinically recognized (Wilcox et al., 1988). This condition became generally recognized when rapid and sensitive HCG assays were introduced into clinical practice, but initially an elevated HCG value in young women without evidence of pregnancy was often classified as a false-positive test (Seppälä et al., 1980). Early pregnancy loss, which also is called ‘biochemical pregnancy’ (Walker et al., 1988), is now well recognized. This condition is associated with lower than expected HCG levels, and assay of HCG in serum is used to identify it especially in connection with ART (Figure 5).

Rapid identification of early pregnancy loss is of value in ART. A failing pregnancy is usually associated with a slower than normal increase in serum HCG, which gradually turns into a decrease (Korhonen et al., 1994). Pregnancy outcome can be predicted with fairly high accuracy using a single HCG determination in serum 12–16 days after embryo transfer (Schmidt et al., 1994; Poikkeus et al., 2002). A threatening abortion can be identified more accurately by serial determinations of serum HCG, and the patients are usually highly motivated to participate in intense monitoring. The increase in serum HCG level is exponential, i.e. the concentrations double in ~1.5–2 days in successful pregnancies. However, the rate of increase varies considerably between individuals. On the basis of the range of increase rates observed, various formulas and algorithms for identification of early pregnancy loss have been developed. In a viable pregnancy achieved by IVF, the median increase in HCG is 50% per day and 124% in 2 days (Barnhart et al., 2004). For the identification of a threatening abortion, the slowest increase in a successful pregnancy is important. The minimal daily increase in symptomatic patients with a viable pregnancy is >24% and that in 2 days 53%. A slower increase is thus a strong indication for an imminent abortion (Barnhart et al., 2004).

In ectopic pregnancies achieved by IVF, the increase in serum HCG is on average delayed by 1.5 days, but the rate of increase is usually normal for the first 4 weeks after embryo transfer. This indicates that implantation is delayed (Korhonen et al., 1996). Determination of HCG in combination with sonography is used to
detect complications later in pregnancy. In a normal pregnancy, an intrauterine sac becomes visible with vaginal sonography by 33 days of gestation when serum HCG is 500–1000 IU/l. A yolk sac is visible by day 38 and fetal heart motion by day 43 (Cacciatore et al., 1990). Failure to detect an intrauterine fetal sac when HCG reaches a certain ‘discriminatory zone’, which initially was defined as 6000–7000 IU/l (Kadar et al., 1981), indicates that the pregnancy is ectopic. With a threshold of 6500 IU/l, the predictive value of sonography approaches 100%, but with improved sono-graphic techniques such as vaginal sonography, thresholds as low as 1000 IU/l have been successfully used (Cacciatore et al., 1989). However, the reliability of this approach is highly dependent on sonographer skill, and discriminatory zones of 1000–2000 IU/l may be too low for general use (Condous et al., 2005).

Ectopic pregnancy is mostly treated surgically or with methotrexate, but some patients recover spontaneously and can be treated conservatively. These patients are monitored by assay of serum HCG, and sinking levels indicate spontaneous resolution. The likelihood of spontaneous resolution is inversely related to the HCG level on admission, but there is no threshold below which success is guaranteed, and rupture of an adnexal mass may occur even when HCG has decreased to 25 IU/l (Korhonen et al., 1994; Murray et al., 2005). Methotrexate treatment is an option in selected patients whose serum level is <5000 IU/l. After treatment, the HCG level is monitored at weekly intervals until the level drops to <15 IU/l (Murray et al., 2005). Women with known risk factors of ectopic pregnancy can be screened by sonography and assay of serum HCG. By this approach, an ectopic pregnancy can be detected on average at a gestational age of 37 days, which in most cases is before development of clinical symptoms (Cacciatore et al., 1994). This facilitates early tube-sparing therapy.

**Screening for Down’s syndrome**

Pregnant women carrying a child with trisomy 21 (Down’s syndrome) have elevated serum concentrations of HCG (Bogart et al., 1987) and HCGβ (Macri et al., 1990). Determinations of HCG or HCGβ together with α-fetoprotein (AFP), inhibin-A and estriol in maternal serum have been widely used for second trimester Down’s syndrome screening, whereas determination of HCGβ and pregnancy-associated plasma protein-A (PAPP-A) in combination with measurement of nuchal translucency (Wald et al., 2005) is used for first trimester screening. In a recent multicentre study, screening with these markers during the 11th, 12th and 13th weeks of pregnancy gave detection rates of 87, 85 and 82%, respectively, with a 5% rate of false-positive results. Second trimester screening at 15–18 weeks of pregnancy with AFP, HCGβ, estriol and PAPP-A in combination with age gave a detection rate of 81%. Even higher detection rates were obtained with a combination of first and second trimester screening (Malone et al., 2005).

Determination of HCG in urine has been reported to provide substantially better discrimination between Down’s syndrome patients and controls than that of HCG (Cole et al., 1999), but the use of urine samples has not gained acceptance and, when determined in serum, HCG has not been found to provide any advantage over HCGβ (Palomaki et al., 2005). Recently, HCG in maternal urine of mothers carrying a fetus with Down’s syndrome has been found to have a reduced content of sialic acid, but the clinical value of this finding remains to be determined (Sutton and Cole, 2004).

**Use of HCG assays for diagnosis and monitoring of cancer**

**Gestational trophoblastic disease**

HCG is an extremely sensitive and specific marker for gestational trophoblastic disease (GTD) and for some germ cell tumours of the testis. In GTD, the sensitivity and specificity of HCG approach 100%, while about 50% of the patients with testicular germ cell tumours have elevated HCG concentrations. Furthermore, isolated elevation of HCGβ occurs in 30–70% of patients with various non-trophoblastic tumours (Stenman et al., 2004).

GTD comprises a spectrum of tumours ranging from hydatidiform mole to choriocarcinoma. Choriocarcinoma often develops after a molar disease, and the risk associated with a complete mole is higher than after an incomplete mole. GTD may also develop after an ectopic pregnancy and occasionally in women without evidence of clinical pregnancy (Seckl et al., 2000; Seki et al., 2004). Placental site trophoblastic tumour is a rare and therapy-resistant type of choriocarcinoma that develops after a pregnancy (Baergen et al., 2006).

Trophoblastic tumours containing only ∼100 000 cells cause elevated serum concentrations of HCG, which thus is by far the most sensitive tumour marker known. A relapse of GTD is usually detected on the basis of an increasing serum HCG before the tumour is large enough to be detected by any other method. Therefore, therapy of a relapse is directed on the basis of the HCG value (Bagshawe, 1992). Placental site trophoblastic tumours often produce little HCG and one-fourth have been reported to be HCG negative (Baergen et al., 2006).

Assays measuring HCG and HCGβ together are mostly used to monitor patients with GTD, but separate assays for these two facilitate differentiation between benign and malignant trophoblastic
diseases. In patients with molar disease, the proportion of HCGβ is larger than in pregnant women (Gaspard et al., 1980; Ozturk et al., 1988), and patients with choriocarcinoma have a still higher proportion. The percentage is dependent on the assays used, but a proportion based on molar concentrations >5% has been found to be strongly associated with malignant GTD (Stenman et al., 1985; Berkowitz et al., 1989; Khazaie et al., 1989). Furthermore, a high proportion of HCGβ is a sign of aggressive disease, and a rising proportion has been found to predict the development of therapy resistance (Vaitukaitis and Ebersole, 1976; Vartiainen et al., 2002). HCGβ disappears from circulation more slowly than HCG (Korhonen et al., 1997), and the proportion increases after successful therapy (Vartiainen et al., 1998, 2002). Therefore, the proportion of HCGβ should be evaluated before therapy or during a relapse (Figure 6).

Gonadal germ cell tumours
Testicular germ cell tumours are of two main types, seminomas and non-seminomatous germ cell tumours (NSGCTs). Elevated serum HCG concentrations are observed in ~50% of patients with NSGCTs and 10–15% of those with seminoma. However, up to 50% of the latter may produce HCGβ (Hoshi et al., 2000; Stenman et al., 2004). Ovarian germ cell tumours are rare, but those containing trophoblastic components usually produce HCG (Stenman et al., 2004).

Non-trophoblastic cancers
With the first specific RIAs for HCG, elevated immunoreactivity was observed in serum from a large proportion of patients with non-trophoblastic tumours (Braunstein et al., 1973) including gynaecological cancers (Donaldson et al., 1980). However, changes in the levels did not reliably reflect the course of the disease (Rutanen and Seppala, 1978). This was probably explained by the increased level of serum HCG derived from the pituitary caused by ovariectomy (Stenman et al., 1987), which often is part of the treatment of gynaecological cancers. It is now known that increased HCG immunoreactivity in serum from patients with non-trophoblastic cancer is caused by expression of HCGβ by the tumours. Elevated serum levels occur in 30–70% of patients with most types of non-trophoblastic cancer, and this is mostly associated with adverse outcome. This topic has recently been reviewed (Stenman et al., 2004).

Gynaecological cancers
Many of the first studies on ectopic expression of HCG-like immunoreactivity in cancer were performed on cervical and ovarian cancer cell lines (Braunstein et al., 1978; Hussa et al., 1978; Ruddon et al., 1980) and patients with various gynaecological cancers (Donaldson et al., 1980; Cauchi et al., 1981; Grossmann et al., 1995). Several cervix cancer cell lines have been shown to produce HCGβ (Story et al., 1981; Nozawa et al., 1983; Hussa et al., 1986), which was also found in tumour extracts (Papapetrou and Nicopoulou, 1986). When specific assays were developed, it could be shown that elevated levels of HCGβ and HCGβcf occur in urine of patients with cervical, endometrioid and ovarian cancers (Cole et al., 1988). Some of the patients with cervical cancer and high urinary levels of HCGβcf had elevated HCG immunoreactivity in serum (Norman et al., 1990). Later studies with sensitive and specific assays confirmed that the HCG-like immunoreactivity in the serum of patients with non-trophoblastic tumours consists of HCGβ (Alfthan et al., 1992b; Marcillac et al., 1992). Elevated levels of HCGβcf have even been detected in urine of some patients with cervical intraepithelial neoplasia (Norman et al., 1993), and clearly elevated levels are associated with adverse outcome of cervical (Carter et al., 1994; Crawford et al., 1998) and vulvovaginal cancers (Carter et al., 1995). In ovarian cancer, an elevated serum level of HCGβ has been shown to be a strong prognostic factor that is independent of stage and grade (Vartiainen et al., 2001).

False-positive HCG results
Because HCG is a very sensitive tumour marker when pregnancy has been excluded, an elevated HCG level in serum is a strong indication for a trophoblastic or a germ cell tumour. Therefore, patients without any other evidence of cancer but with a falsely elevated HCG value have been inappropriately treated with chemotherapy, which occasionally has caused severe complications (Rotmensch and Cole, 2000). It is therefore important to be aware of this possibility and to know how to identify such results. The causes of false- and apparently false-positive results are listed in Table IV. This topic has recently been reviewed (Braunstein, 2002).

Most false-positive results are caused by antibodies to animal immunoglobulins (Igs) in the serum of the patients. So-called heterophilic antibodies to animal Igs occur in the blood of everyone. They are probably induced by ingestion of cow’s milk and other foodstuff of animal origin or by contact with animals. Rheumatoid factors are anti-Ig antibodies that also may react with animal antibodies. Virtually, all HCG assays are presently sandwich assays using a capture antibody bound to a solid phase and a detector antibody that is labelled, usually with a fluorophore, an enzyme or a chemiluminescent substance. Most assays use mouse monoclonal antibodies

**Figure 6.** Serum HCG and HCGβ during successful therapy with hysterectomy and chemotherapy (hatched blocks) of a metastatic choriocarcinoma. The horizontal broken lines show the upper reference limits for HCG (thick line) and HCGβ (thin line). Because HCGβ disappears more slowly from circulation than HCG, the ratio between these increases with time. After the initial decrease, serum HCG increases again to ~8 pmol/l (which is within the reference range) due to chemotherapy-induced gonadal suppression. Carcinoma was diagnosed about 300 days after application of a Levonova contraceptive device.
Causes of false-positive and apparently false HCG results

<table>
<thead>
<tr>
<th>False results caused by interfering substances</th>
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<tbody>
<tr>
<td>Anti-animal immunoglobulin antibodies</td>
</tr>
<tr>
<td>Heterophilic antibodies</td>
</tr>
<tr>
<td>Species-specific antibodies (HAMA)</td>
</tr>
<tr>
<td>Rheumatoid factors</td>
</tr>
<tr>
<td>Complement</td>
</tr>
<tr>
<td>Non-specific serum factors</td>
</tr>
<tr>
<td>Anti-HCG antibodies</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Assay issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exchange of sample</td>
</tr>
<tr>
<td>Carryover by pipetting device</td>
</tr>
<tr>
<td>Contamination affecting label detection</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Causes of apparently false-positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated levels of pituitary HCG</td>
</tr>
<tr>
<td>Perimenopause</td>
</tr>
<tr>
<td>Chemotherapy-induced suppression of gonadal function</td>
</tr>
<tr>
<td>Previous injection of HCG</td>
</tr>
</tbody>
</table>

Both as capture and as detector antibodies. The capture antibody catches HCG from the sample, and the detector antibody binds to another epitope on HCG, forming a sandwich. The concentration of HCG is directly proportional to the amount of bound detector antibody. An anti-animal Ig in the sample will give rise to a sandwich by bridging capture and detector antibodies causing a false-positive result. Because Igs from various species are highly homologous, the same antibody can react with Igs from different animals. Thus, the use of catcher and detector antibodies from different species, e.g. mouse and rabbit, does not eliminate the problem. Assay manufacturers are aware of this problem and add Igs that block the interferences. However, some patient sera contain such high concentrations of heterophilic antibodies that the blocking is insufficient and a false-positive result is obtained.

Mouse antibodies are occasionally used for tumour imaging and therapy. This often gives rise to human anti-mouse Ig antibodies (HAMA), which cause false-positive results in the same way as heterophilic antibodies, and their effect can also be blocked by the addition of mouse and other animal Igs to the assay buffer. Despite this, a small proportion of the samples will contain more HAMA or heterophilic antibodies than the added Igs are capable of neutralizing. This will lead to a false-positive result, and it is important to keep this possibility in mind and to perform additional studies necessary to identify the condition. If the sample is available, the effect of heterophilic antibodies can be inhibited or at least reduced by the addition of mouse Ig or mouse serum to the sample (Turpeinen et al., 1995). Dilution of the sample with buffer (or rather the 0-standard) usually results in a lower-than-expected result. Another useful check is to analyse the sample with another method: the degree of interference is virtually always different in different methods (Cole et al., 2004). It is also advisable to take a new sample. This reveals whether the result is caused by exchange of the sample. A false-positive result is usually similar in a new sample taken within 1–2 weeks, whereas in cancer the concentrations usually increase with time. At the same time, a urine sample should be obtained. Igs are not excreted into urine, and a negative HCG result in urine is a reliable sign of a false-positive serum result (Stenman et al., 2004). If the HCG level is only moderately elevated, a negative result in urine may be caused by a very dilute urine, which is recognized by the determination of urine density. In urine with an average density (1.015), the HCG concentration is about 50% of that in serum but the variation is large.

If the elevated HCG immunoreactivity in serum is caused by HCGβ, its concentration in urine is usually much lower than in serum because most of HCGβ is degraded to HCGβcf during excretion into urine (Alfthan et al., 1992b), and most HCG assays do not measure HCGβcf (Cole et al., 2004).

Various complement factors react with antibodies, but the effect is highly dependent on the isotype of the antibody used. Thus, mouse IgG2 often reacts with complement. In a sandwich assay, the effect is opposite to that of heterophilic antibodies, i.e. the result is too low because complement reduces the binding capacity of the antibody (Bormer, 1989). In an inhibition assay, complement will cause a false-positive result. Because of this, most assay manufacturers avoid using IgG2 antibodies. Serum also contains other factors that non-specifically block the antigen–antibody reaction. This effect can be detected as a higher-than-expected result if the sample is diluted in a buffer devoid of protein. This ‘matrix effect’ is usually taken into account by preparing the calibrators in serum devoid of the analyte. However, because the content of interfering factors varies from one sample to another, there is always between-method variation, which is most visible at low concentrations.

Therapeutic use of urinary HCG has in rare cases caused induction of antibodies (Braunstein, 2002). This will cause a false low result in sandwich assays but falsely elevated results in inhibition assays. With previous models of automatic analyser, carryover of HCG from a pregnancy sample could cause falsely elevated result, but this is presently a rare problem. Contaminants causing errors in label detection is a potential problem in so-called homogeneous assays, i.e. assays without separation of free and bound label. Presently, this assay principle is very rarely used in HCG assays and thus it is not a practical problem.

The HCG concentrations in serum increase in the menopause. With sensitive and specific methods, the highest levels observed are 7–8 IU/l, but with other methods, levels up to 15 IU/l (Snyder et al., 2005) and even 25 IU/l have been observed (Braunstein, 2002). Chemotherapy used to treat malignant tumours often induces gonadal suppression and elevation of serum HCG to post-menopausal levels (Stenman et al., 2004). This is important when young women treated for trophoblastic tumours and males treated for testicular cancer are monitored by the determination of serum HCG. In these cases, post-menopausal reference limits need to be applied because the serum concentrations often exceed 3–5 IU/l and occasionally reach levels up to 10–15 IU/l. This does not need to indicate a relapse but rather a physiological response to gonadal suppression. The condition can be identified by the measurement of serum LH and FSH, the levels of which are post-menopausal. When necessary, suppression of the HCG level by estrogen replacement can be used to identify this iatrogenic HCG elevation (Stenman et al., 2004).

When HCG treatment is used to induce ovulation, the concentrations in serum increase to 500–1000 IU/l after injection of 5000 IU and decrease to <5 IU/l within 8–12 days (Stenman et al., 1997). Athletes may use HCG to stimulate gonadal steroid production for doping purposes, and this may be the cause of an elevated HCG value (Braunstein, 2002).

HCG isoforms: classification, functions, clinical use
HCG vaccines

Because HCG is necessary for maintenance of pregnancy, neutralization of HCG activity with antibodies can be used for contraception. The immunological tolerance can be broken and antibody formation induced by conjugation of HCG β to a foreign protein such as tetanus and diphtheria toxoids. Repeated vaccination gives rise to high-affinity antibodies that neutralize circulating HCG and provide contraception within 2–6 months. If the antibody concentration exceeds 50 ng/ml, the contraceptive effect approaches 100%, but in one-fifth of the immunized women the response is lower (Talwar et al., 1994). After three immunizations, the antibody titre remains at levels providing contraception for 3–6 months; thus the effect is reversible. Owing to the variable duration of contraception, monitoring of the antibody titre is necessary. The antibodies cross-react with LH, but despite this, the endocrine function and menstrual cycles remain normal (Talwar et al., 1994). Formation of antibodies to LH can be eliminated by immunizing with the unique CTP of HCG conjugated to diphtheria toxoid (Jones et al., 1988). However, the antibodies obtained by this approach are of relatively low affinity, and extensive clinical studies have not been performed (Nazz et al., 2005). Contraceptive vaccines based on HCG immunization are not in clinical use.

Because many tumours produce HCG β, vaccination with HCG is also of potential utility as an antitumour therapy. A significant increase in survival has been reported after immunization of patients with advanced colorectal cancer with a vaccine containing a 39-amino-acid part of CTP conjugated to diphtheria toxoid (Moulton et al., 2002).

Many groups are presently developing alternative HCG-based vaccines, but so far results of clinical studies have not been reported. Formation of antibodies cross-reacting with LH may be avoided by immunizing with rHCG, in which one amino acid, arginine 68, has been replaced with glutamic acid. This HCG variant has been successfully used to immunize animals, but it remains to be shown whether it is useful in humans.

Conclusions

Detection of HCG forms the basis of pregnancy tests, and quantitative determinations of various forms of HCG are used for diagnosis and monitoring of pregnancy complications such as early pregnancy loss, ectopic pregnancy and fetal trisomy 21. Various forms of HCG can also serve as markers for gestational trophoblastic tumours, testicular cancer and many non-trophoblastic tumours. In GTD, the concentrations of HCG in serum and urine are virtually always elevated and the use of HCG assays is mandatory for monitoring of the disease. In germ cell tumours of the testis, HCG is a useful marker in about half of the patients, and in many non-trophoblastic cancers, HCG β is a strong and independent prognostic marker. Utilization of HCG β as a tumour marker is hampered by limited availability of sensitive and specific assays.

Because serum HCG is such a reliable tumour marker, a false-positive result has occasionally been thought to be caused by an occult cancer, and this has led to inappropriate therapy. It is important to recognize this possibility and not treat ‘the HCG result’ but the patient.

HCG plays a central role in ART, and in addition to its use to induce ovulation, it has recently been found to also improve the receptivity of the endometrium. Immunization with HCG, or parts of it, conjugated to immunostimulatory proteins induces antibodies that produce reversible contraception by neutralizing HCG in circulation. Despite promising results, this approach is not in clinical use. Vaccination with HCG β is also used for experimental tumour therapy.

References


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