Infections and sepsis are among the most common reasons for neonatal morbidity and mortality. Early diagnosis is difficult because clinical presentation is highly variable and signs are often subtle and common to a variety of conditions. Among the proposed early indicators of infection and sepsis are serum concentrations of interleukin (IL)-6, IL-8, and IL-10. It is believed that IL-8 is a sensitive indicator of infection and that high concentrations of IL-6 and IL-10 are indicators of sepsis and predictors of mortality (1–3). The concentrations of each of these cytokines in serum vary by several orders of magnitude (1–3). Literature-reported cutoff values for IL-8 are >70 ng/L (2) or >18 ng/L (1) for infection, and values >10 000 ng/L have been reported (1). IL-6 >175 ng/L is predictive of sepsis, and values >747 ng/L are predictive for pneumonia (3). IL-6 is also believed to be predictive of necrotizing enterocolitis (3). IL-10 >420 ng/L correlates with neonatal death (3).

The ELISAs commonly used for cytokine detection require 50–100 μL of serum (~100–200 μL of peripheral blood in the neonate) per cytokine. To determine the stage of an infection, measurement of several cytokines at multiple time points can be of importance (3). Combining pro- and antiinflammatory cytokines in a single assay yields an overall view on the patient’s inflammatory status; may allow differentiation among infection, sepsis, and enterocolitis; and thus may improve diagnostic accuracy. In neonates, however, particularly preterm neonates, such combined measurements are often hampered by lack of sufficient obtainable blood (1). Furthermore, although the ELISAs are adequate for measuring these cytokines, they often require multiple dilutions to cover a wide concentration range because their dynamic range is only ~2–3 logs.

Recently, fluorescent bead array assays, such as the Cytometric Bead Array™ (CBA; BD Biosciences, San Jose, CA) (4), that involve measurement by flow cytometry have become available, and they have widened the assay dynamic range greatly (4, 5). This in turn improved efficiency by decreasing the required number of dilutions. These assays are multiplexed such that numerous substances are measured simultaneously in a single well. The CBA assay consists of a mixture of six types of beads uniform in size but containing different fluorescence intensities of a red-emitting dye. A different capture antibody (Ab) against one of six cytokines is covalently coupled to each type of bead. Cytokines bound to these Abs are detected by use of Abs labeled with phycoerythrin. The fluorescence intensity measured with phycoerythrin is proportional to the cytokine concentration in the sample and is quantified from a calibration curve. The wider dynamic range is attributable to the use of fluorescence detection, which has a range of 4–5 logs, and the highly efficient capturing capability of the Ab-linked particles (4). Particles in suspension are much more efficient than the static surface of ELISA well bottoms in capturing antigens. We applied this system to pediatric samples and compared the results with those of standard ELISA analyses.

The study was approved by the ethics committee of the
University of Leipzig. We collected peripheral blood samples from children (age range, 2 weeks to 16 years) who had undergone cardiovascular surgery with cardiopulmonary bypass. Written informed consent was obtained from the parents of all 19 children who participated in the study. This group of patients was chosen because of the substantial intraoperative changes of IL-6, -8, and -10 known to occur during surgery with cardiopulmonary bypass (6). From each patient, depending on body weight, we collected 0.1–1.0 mL of peripheral blood at up to 10 different time points. We also obtained postoperative drainage fluids from the pericardium or the pleura from five of the patients as high IL-6 concentration samples (7). A total of 187 samples were collected and centrifuged at 2500g for 10 min; the supernatants were collected and stored in aliquots at −80 °C until analysis.

Cytokines were quantified by ELISA (all from R&D Systems) and the CBA (BD Biosciences) method. With the ELISAs, determination of all three cytokines required 200 μL of serum and incubation time was 4–5 h. The CBA assay, which simultaneously quantifies IL-1β, IL-6, IL-8, IL-10, IL-12p75, and tumor necrosis factor-α in a single sample, uses 50 μL. To save serum, we modified the CBA assay by reducing the serum volume to 25 μL/sample. Incubation time was 3 h. The fluorescence produced by the CBA beads was measured on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with its software (4). For the ELISAs, the lowest and highest calibrators were 3.1 and 300 ng/L, respectively, for IL-6; 7.8 and 2000 ng/L for IL-8; and 7.8 and 500 ng/L for IL-10. For the CBA, the range of calibrators was extended from the original 20–5000 ng/L to 5.0–50 000 ng/L for all cytokines. For the ELISAs, intra- and interassay imprecision (as CVs) was 2% and 3%, respectively, for IL-6 at 100 ng/L; 5% and 7% for IL-8 at 200 ng/L; and 4% and 8% for IL-10 at 80 ng/L. For the CBA, intra- and interassay imprecision was 6% and 8%, respectively, for IL-6 at 80 ng/L; 4% and 4% for IL-8 at 80 ng/L; and 6% and 8% for IL-10 at 80 ng/L.

With the CBA, the calibration range for each analyte was at least 10 times wider than the calibration range for the corresponding ELISA (Fig. 1). The detection limits (based on the value obtained for the zero calibrator +3 SD) for IL-6, IL-10, and IL-8 were <5 ng/L for both the ELISAs and the CBA. This showed that both assay types are similarly able to detect serum cytokine concentrations at the reported cutoff concentrations (1–3). The two assays yielded concentrations that were virtually identical, as exemplified by the characteristic time courses of two patients (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue6/). The values obtained by the ELISA and CBA were highly correlated over five decades of log concentration difference (Table 1). The mean (SD) concentrations in the drainage fluids were high for IL-6 [ELISA, 24 003 (13 300) ng/L; CBA, 24 900 (9075) ng/L], as expected (7), and for IL-8 [ELISA, 3050 (3160) ng/L; CBA, 3273 (3928) ng/L], but the mean concentration of IL-10 was intermediate [ELISA, 71 (39) ng/L; CBA, 83 (46) ng/L]. With the CBA, no dilutions were needed to measure the high concentrations in the drainage fluids, but several dilution steps were needed with the ELISAs. We obtained sufficient sample material to measure IL-6, but not IL-8 and IL-10, in all 187 samples by ELISA (Table 1). With the CBA and ELISAs, the concentrations of IL-1β, IL-12p75, and tumor necrosis factor-α were near the concentration of the lowest calibrator and did not significantly change with time, as expected (5) (data not shown); the concentrations of these analytes were also in this range in the drainage fluids.

Cytokine measurement by multiplexed immunoassay and flow cytometry promises to be of substantial benefit for the critically ill child. The amount of blood required is reduced by more than 80% for the three cytokines and may be further reduced. The results highly correlate with the results obtained by the ELISAs used for routine diagnostic purposes. On the basis of our data, the CBA assay is particularly convenient in the presence of active infection, when cytokine concentrations are quite high. Because flow cytometers are widely used and understood in clinical laboratories, adaptation of the CBA into clinical routine may be readily accomplished. The system is flexible and cost-effective because it requires less manual operating time than several ELISAs and avoids additional dilutions for high-concentration samples. New bead populations that measure additional markers may be added to increase diagnostic capability, and markers that are not useful in a specific situation can be removed to reduce costs (5). The critically ill child most likely will benefit from these developments.

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## Table 1. Correlation between cytokine concentrations.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Equation*</th>
<th>Regression line</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>y = -3.71 (2.94) + 1.183 (0.019)x</td>
<td>P &lt;0.0001 S_{yx} 27.72 r 0.99 n 104</td>
</tr>
<tr>
<td>IL-6</td>
<td>y = 70.29 (80.78) + 0.944 (0.018)x</td>
<td>P &lt;0.0001 S_{yx} 1091.07 r 0.97 n 187</td>
</tr>
<tr>
<td>IL-8</td>
<td>y = -4.81 (26.63) + 0.808 (0.024)x</td>
<td>P &lt;0.0001 S_{yx} 338.83 r 0.93 n 173</td>
</tr>
</tbody>
</table>

* x, values determined by ELISA; y, values determined by CBA.
The Serum Growth Hormone (GH) Response to Provocative Tests Is Dependent on Type of Assay in Autosomal Dominant Isolated GH Deficiency because of an ARG183HIS (R183H) GH-I Gene Mutation, Roxana Marino, Eduarda Chater, Monica Warn, Maria Giacchi, Esperanza Berensztein, Marco A. Ricarola, and Alicia Belgorosky (Research Laboratory and Endocrinology Service, Garrahan Pediatric Hospital, Buenos Aires, Argentina 1245; * address correspondence to this author at: Coordinación de Investigación, Hospital de Pediatría Garrahan, C. de los Pozos 1881-P2, Buenos Aires, Argentina 1245; fax 5411-4308-5325, e-mail abelgo@elsito.net)

The clinical appearance and genetic basis of familial isolated growth hormone deficiency (IGHD) are heterogeneous and are associated with at least four types of Mendelian disorders: two forms with autosomal recessive inheritance (IGHD type 1A and 1B), one with autosomal dominant inheritance (IGHD type 2), and one with X-linked inheritance (IGHD type 3) (1, 2).

Recently, Deladèo et al. (3) reported a new form of IGHD type 2 in four unrelated families. These patients carried an ARG183HIS (R183H) GH-I mutation. On the basis of in vivo and in vitro experiments, the authors reported that the R183H mutant GH peptide severely impaired GH-regulated secretion. Furthermore, this GH mutant was found to have an effect on GH receptor/GH-binding protein (GHR/GHBP) transcription identical to that of the 22-kDa GH when tested in a human hepatoma cell line (4).

Human GH represents a family of proteins rather than a single hormone. Indeed, GH is among the more heterogeneous polypeptide hormones, and the proportions of immunoreactive forms in plasma vary with time after a GH secretory pulse (5). The simultaneous use of an immunoassay that detects most serum GH isoforms (total GH) and one specific for the 22-kDa GH form makes it possible to uncover changes in the pattern of secretion of GH isoforms. Moreover, discrepancies between GH immunoologic and biological activities have been reported (6). It has therefore been proposed that the ratio of non-22-kDa GH to 22-kDa GH isoforms may have important implications for normal and abnormal growth (7).

We studied two nonrelated patients from Argentina with severe short stature and IGHD type 2 attributable to the R183H GH-I gene mutation and found that the evaluation of GH response to provocative tests might be misleading in these patients, depending on the type of assay used to assess GH secretion.

Serum GH was measured by two commercial immunoassays: a polyclonal IRMA, which uses WHO International Reference Preparation (IRP) human GH for RIA 66/217 (SER 66/217); and DELFIA, a time-resolved immunofluorometric assay, which uses WHO IRP for human GH for bioassay 80/505 (DELFIA 80/505). The intra- and interassay CVs were 5% (n = 90) and 7% (n = 90), respectively, for the SER 66/217 assay (range, 0.69–17.0 μg/L) and 3.3% (n = 90) and 4.7% (n = 90), respectively, for the DELFIA assay (range, 0.25–10.9 μg/L). As reported previously, the cutoff values for provocative tests in children differed markedly depending on the assay: 10 μg/L for the SER 66/217 and 4.81 μg/L for the DELFIA 80/505 (8). One reason for this discrepancy is that the SER 66/217 uses an antibody recognizing multiple GH epitopes present in several molecular forms in serum, whereas the DELFIA 80/505 is specific for 22-kDa GH (5). Indeed, cross-reactivity of 20-kDa GH in the 22-kDa GH assay was <0.001% (9). Conversion factors have been proposed to calculate equivalent values between polyclonal and monoclonal assays for serum concentrations in adults (10) and children (8). We propose designating SER 66/217 serum values as serum “total” GH and DELFIA 80/505 values as serum 22-kDa GH. We studied GH secretion, using pharmacologic stimulation with arginine and clonidine, as described previously (11).

Serum GH bioactivity was analyzed in the Nb2 bioassay according to a modification of the method published by Radetti et al. (12), using the CellTiter 96® AQüous Non-Radioactive Cell Proliferation Assay (Promega Corporation).

Intra- and interassay CVs were 11% (n = 10) and 21% (n = 16), respectively, for the GH bioassay (range, 0.5–39.1 μg/L). Normal response of bioactive GH to arginine and clonidine tests was defined in 21 control children (14 males and 7 females) 0.48–13.8 years of age with moderate short stature [–0.75 ± 1.32 standard deviation score (SDS)] and normal 22-kDa GH responses to the tests. Mean (SD) GH response was 10 (3.1) μg/L (95% confidence interval, 4.02–16.0 μg/L). The cutoff for bioactive GH response was set at the lower limit of the 95% confidence interval (mean ± 1 SD × 1.96) of the control group. This value was 4 μg/L.

Serum insulin-like growth factor-I (IGF-I) was measured with the IGF-I generation test as described previously (13). Response was considered normal when the increase in serum IGF-I was ≥15 μg/L (14, 15). It was also compared with that of nine prepubertal patients (median age, 6.3 years; range, 3.7–11.2 years) with idiopathic GH deficiency.

References