Pediatric Reference Ranges for Proinflammatory and Anti-Inflammatory Cytokines in Cerebrospinal Fluid and Serum by Multiplexed Immunoassay

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To define cytokine concentrations and detectability in children with noninflammatory neurological disorders (NIND). The multiplex bead assay technology was used for simultaneous measurement of 34 soluble cytokines/chemokines in cerebrospinal fluid (CSF) from 73 NIND. Sera from 36 healthy children and 37 NIND also were analyzed. In CSF, CXCL10 had the highest concentration; CCL2, CXCL10, and interleukin (IL)-6 were detectable in all samples, and CXCL8, CCL22, CXCL1, IL-16, and IL-1 receptor antagonist were found in $\geq 50\%$ of the samples. In serum, CXCL1 had the highest concentration; sIL-2Ra, CXCL1, CXCL10, and CCL22 were detectable in all samples, and CCL2, IL-12, CCL5, and granulocyte monocyte colony-stimulating factor (GM-CSF) were found in $\geq 50\%$ of the samples. The mean CSF:serum ratio for CCL2 was several-fold higher than the rest, with the CXCL10 and CXCL8 ratios also > 1 . Intercorrelations between CSF cytokines included CCL2 versus CXCL8 and IL-6, and CXCL1 versus CCL22, reflecting both T-helper-1 (Th1)/Th1 and Th1/Th2 relations. Serum correlations included CCL11 versus CCL2, GM-CSF, and IL-4. For serum cytokines, the agreement between healthy children and NIND was good, with the exception of higher CCL4 in NIND. Cytokines in children varied greatly in concentration and detectability, with chemokines predominating in the CSF. These data allow investigators to select their own kit cytokines, instead of manufacturer-selected cytokines, for greater cost-effectiveness and interpretability.

Introduction

STUDIES OF CYTOKINES in children with inflammatory neurological disorders are critically dependent on controls. In cerebrospinal fluid (CSF) studies, noninflammatory neurological disorders (NIND) typically are used as controls because of ethics issues involved in performing lumbar punctures on healthy children (Pranzatelli and others 2012). In serum studies, however, healthy children and NIND can be compared (Pranzatelli and others 2008). Reference ranges for CSF cytokines and chemokines have not been established in a larger group of children, which was one of the goals of the present report.

The multiplex bead assay technology affords the opportunity to evaluate the cytokine/chemokine network in CSF, serum, or plasma from inflammatory and infectious disorders (Mandy and others 2001). The analytes are captured to spectrally distinct microbeads and tagged with fluorescent-labeled markers, which are excited and detected by flow cytometry (Khan and others 2004). Although the technique is expensive, it is powerful, and a multitude of analytes can be

measured simultaneously in a small sample volume (Nattelson and others 2005). Manufactured kits, available from several vendors, lend themselves to use as a screening or discovery tool.

Multiplex bead-based assays may generate a daunting amount of cytokine data that must be interpreted. Cytokines, which are low-molecular-weight proteins, can be grouped by families, such as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), and colony-stimulating factors. A traditional way to conceptualize cytokine function has been to differentiate proinflammatory versus anti-inflammatory cytokines, though the function of some changes with the immunological milieu (Kerschensteiner and others 2009). Pivotal cytokines drive the immune system toward a T-helper-1 (Th1) or Th2 response (Sredni-Kenigsbuch 2002), though the Th1-versus-Th2 paradigm is simplistic (Wan 2010). Proinflammatory cytokines include interferon-gamma (IFN- γ), IL-2, and IL-12, and monokines IL-1 β , IL-6, IL-8, and tumor necrosis factor-alpha (TNF- α) (Martins and others 2011; Choi and others 2011). From the perspective of Th1-driven inflammation, anti-inflammatory and immunoregulatory

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cytokines include IL-4, IL-5, eotaxin (CCL11), IL-10, IL-13, and transforming growth factor- β (Martins and others 2011), and soluble receptor antagonists (-Ra) of inflammatory cytokines downregulate inflammation, too (Bartfai and others 2007).

Of particular interest are cytokines that induce chemokines, which are not usually expressed in nonlymphoid tissues under physiological conditions. Classification of chemokines, or chemoattractant cytokines, based on structure (such as C-C or C-X-C motif) has superseded older common names, though they are known by both (Zlotnik and Yoshie 2000). Chemokine ligands (L) can be conceptualized in the context of superfamilies and receptors they bind (R), such as CXCL1 and CXCL8 both binding to CXCR2; CCL2 to CCR2; CCL4 to CCR1 and CCR5; CCL11 to CCR3; and CCL22 to CCR4 and CCR6 (Zlotnik and Yoshie 2000). CXCR3 and CCR5 are linked to a Th1 phenotype; CCR3, 4, and 8 to a Th2 phenotype (Zlotnik and Yoshie 2000).

Patients and Methods

Patients

After recruitment of children to the National Pediatric Myoclonus Center from 2000 to 2008, parents signed consent for this Institutional Review Board-approved study. Lumbar punctures, performed under standardized procedures (Pranzatelli and others 2012), yielded CSF for the cytokine studies from 73 children with various NIND (Table 1). The mean age was 8.2 ± 4.1 years (range 0.94–18 years). There were 42 boys and 31 girls. Serum was obtained from 36 healthy children and 37 of the same children with NIND. Criteria for establishing specimen suitability have been described (Pranzatelli and others 2012).

Multiplexed fluorescent bead-based immunoassays

Blood collected in serum separator tubes was centrifuged, and serum was pipetted into aliquots, frozen, and stored at -80°C until use. CSF was collected on ice and stored as aliquots. Using a combination of 22-plex and 12-plex Beadlyte Human Cytokine Detection kits (Upstate, Lake Placid, NY), 34 cytokines were measured by multiplexed fluorescent bead-based immunoassay detection (Luminex100 LabMAP system; Luminex Corporation, Austin, TX). Cytokine assays

were performed on batched samples in triplicate as described previously (Pranzatelli and others 2011). Each 96-well plate accommodated $25\mu\text{L}$ undiluted samples from 24 patients, a blank, and 7 dilutions of standard. Cytokine concentrations were calculated by reference to a standard curve for each cytokine. The curve was derived for each assay from various concentrations of the cytokine standards assayed in the same manner as the patient samples. High values were verified on repeat assays.

The 22-plex kit (lot no. 31676, 27490, 32167, and 33540) already has been validated for CSF by spiking CSF with known amounts of each cytokine and demonstrating recovery in parallel to standard curves (Mandy and others 2001). It contained antibody-conjugated beads for IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, granulocyte monocyte colony-stimulating factor (GM-CSF), IFN- γ , TNF- α , eotaxin (CCL11), monocyte chemoattractant protein-1 (MCP-1, CCL2), regulated upon activation T-cell expressed and secreted (RANTES, CCL5), interferon inducible protein-10 (IP-10, CXCL10), and macrophage inflammatory protein-1 α (MIP-1 α , CCL3). The 12-plex kit (lot no. 27175) was used to detect the sFAS ligand, granulocyte colony-stimulating factor (G-CSF), growth-related oncogene (GRO, CXCL1), IFN- α 2a, IL-1 receptor antagonist (IL-1Ra), sIL-2Ra, IL-9, IL-16, MCP-3 (CCL7), macrophage derived chemokine (MDC, CCL22), MIP-1 β (CCL4), and TNF- β .

Lower detection limits (pg/mL) for the 22-plex kit were as follows: IL-1 α , 0.5; IL-1 β , 0.1; IL-2, 0.2; IL-3, 1.3; IL-4, 0.3; IL-5, IL-6, IL-7, CXCL8, IL-10, 0.1; IL-12(p40), 16.4; IL-12(p70), 1.7; IL-13, 0.4; IL-15, 0.1; TNF- α , 2.0; GM-CSF, 1.0; IFN- γ , 2.9; CCL11, 7.0; CCL2, 3.0; CCL5, 2.7; and CCL3, 29. For the 12-plex kit, they were CCL22, 5.4; sFAS ligand, 8.4; G-CSF, 3.5; CXCL1, 29.5; IFN- α 2a, 5.5; IL-16, 32.6; IL-1Ra, 10; sIL-2Ra, 8.3; IL-9, 1.0; CCL7, 24.2; CCL4, 1.0; and TNF- β , 3.3.

In CSF, the interassay coefficient of variance (CV) for CCL2 was 10.8% ($n=13$), and for CXCL8, it was 13.2% ($n=15$). The intra-assay CV for CCL2 was 9.6% ($n=6$), and for CXCL8, 9.3% ($n=6$). In serum, the interassay CV for CXCL1 was 9.6% ($n=7$), and for sIL-2Ra, 17.8% ($n=8$). The corresponding intra-assay CV for CXCL1 was 3.9% ($n=4$), and for sIL-2Ra, 7.7% ($n=7$).

Statistical analysis

Because of the analytical challenges posed by human cytokine data, the data were analyzed as means [analysis of variance (ANOVA)] and medians (Kruskal–Wallis test), as well as the percentage of specimens with detectable levels of a particular cytokine (chi-square or Fisher exact test). The only true medians, however, were for cytokines without samples that had undetectable levels. In serum, cytokine concentrations in healthy children and those with neurological disorders also were compared by *t*-test or Mann–Whitney test. Pearson and Spearman correlations were tested, and only correlations that remained significant after Bonferroni corrections (α 0.05/ n of comparisons) on both are reported. Correlation plots also were visually inspected. Diagnostic categories of pediatric neurological disorders were compared to determine the cohesiveness as a group. For clarity of presentation, undetectable levels were assigned a value of zero. However, data were analyzed with and without those samples.

TABLE 1. SUBGROUP ANALYSIS

	n	Mean age $\pm$ SD (years)	Sex M:F
Healthy children	36	9.3 ± 4.7	23:13
Neurological disorders			
Ataxia	12	5.1 ± 3.3	9:3
Developmental disorders	13	6.9 ± 4.4	6:7
Headache	13	12.7 ± 3.8	5:8
Movement disorders	13	8.9 ± 4.7	8:5
Seizures	10	6.0 ± 4.9	5:5
Miscellaneous	12	6.6 ± 6.1	9:3

The headache group was significantly older than other NIND ($P=0.002$, ANOVA). There were no significant subgroup differences in sex ($P=0.38$, chi-square).

SD, standard deviation; NIND, noninflammatory neurological disorders; ANOVA, analysis of variance.

FIG. 1. Cytokine concentrations in (A) cerebrospinal fluid (CSF) and (B) serum. Means with standard deviation (SD) in parentheses (larger figure) and medians with interquartile range in parentheses (insert) are ranked. Other medians could not be calculated due to the number of samples with undetectable levels.

Results

CSF cytokines

In CSF (Fig. 1A), there were significant differences between the mean concentrations of cytokines ($P < 0.0001$, ANOVA). In *post hoc* tests ($P < 0.05$), the concentrations of CXCL10 and CCL2 were significantly higher than CXCL8, CXCL1, IL-16, IL-1Ra, IL-6, CCL22, and other cytokines. Five of the eight (62%) CSF cytokines with calculable medians were chemokines. There were also significant differences in medians. The median concentrations of CXCL10 and CCL2 also were significantly higher than the others ($P < 0.0001$).

Only CXCL10 and CCL2 were found in 100% of the CSF samples (Fig. 2). Cytokines detected in $>50\%$ of the CSF samples also included CXCL8, IL-6, CCL22, CXCL1, IL-1Ra, and CCL4. Only 12 of 34 cytokines (35%) were found in $>20\%$ of CSF samples.

The CSF/serum ratios differed significantly for means or medians ($P < 0.0001$) (Table 2). The mean ratio for CCL2, which displayed heterogeneity, was significantly higher than all other ratios. The mean CSF:serum ratios for CXCL10 and CXCL8 were also >1 . Means for 15 cytokines were zero or could not be calculated.

Eight cytokines had calculable median ratios. The median ratio for CCL2 was significantly greater than for CXCL10, IL-1Ra, IL-16, CCL11, and CCL22 ($P < 0.0001$). The median ratios for CXCL10 and CXCL8 were significantly higher than for CCL11 and CCL22.

Significant intercorrelations were found for several CSF cytokines (Table 3). These included Th1 with Th1 cytokines (CCL2, CXCL8, and IL-6), and Th2 (CCL22) with Th1 (IL-12). CSF cytokine concentrations did not correlate with patient age, except for a negative correlation for CCL4. However, the r value was low (-0.21 , $P = 0.005$), and many samples had undetectable levels.

Serum cytokines

In serum (Fig. 1B), there were significant differences between the mean concentrations of cytokines ($P < 0.0001$,

ANOVA). The mean concentration of CXCL1 was significantly higher than the other cytokines. The median concentration of CXCL1 also was significantly higher than all other serum cytokines.

CXCL10, CCL22, CXCL1, CCL5, and sIL-2Ra were detected in 100% of the samples (Fig. 2). In the entire dataset,

FIG. 2. Cytokine detection rates in CSF and serum in the entire dataset. Percent detectability was defined as the percentage of samples in which a given cytokine could be detected. The order of the cytokines displayed is from the highest to lowest CSF detection rate.

TABLE 2. CEREBROSPINAL FLUID:SERUM CYTOKINE RATIOS

Cytokine	Mean ratio $\pm$ SD (median ratio)	Cytokine	Mean ratio $\pm$ SD (median ratio)
CCL2	13.6 $\pm$ 28 (6.1)	IFN- γ	0.04 $\pm$ 0.1 (0)
CXCL10	2.7 $\pm$ 7.5 (0.53)	IL-3	0.03 $\pm$ 0.2 (0)
CXCL8	1.1 $\pm$ 1.0 (0.96)	IL-12(p40)	0.03 $\pm$ 0.1 (0)
IL-6	0.82 $\pm$ 0.9 (0.49)	CCL11	0.03 $\pm$ 0.1 (0)
IL-10	0.53 $\pm$ 1.4 (0)	CCL22	0.02 $\pm$ 0.02 (0.02)
IL-1Ra	0.47 $\pm$ 0.5 (0.37)	CXCL1	0.02 $\pm$ 0.02 (0.02)
CCL4	0.39 $\pm$ 0.7 (0)	IL-2	0.01 $\pm$ 0.05 (0)
IL-16	0.28 $\pm$ 0.4 (0.16)	CCL5	0.007 $\pm$ 0.02 (0)
CCL3	0.24 $\pm$ 0.6 (0)	GM-CSF	0.004 $\pm$ 0.02 (0)
CCL7	0.09 $\pm$ 0.2 (0)		

IL, interleukin.

other cytokines found in >50% of the serum samples included CCL2 and IL-12(p40). Only 17 of 34 (50%) serum cytokines were found in >20% of the samples.

Five of the seven (71%) cytokines with the highest serum concentrations were chemokines. Several serum cytokines were intercorrelated (Table 3). These included Th2 with Th2 (CCL11 and IL-4) and Th1 (CCL2) with Th2 (CCL11 and IL-4). No significant correlations between patient age and serum cytokine concentrations were found, except for CCL4 ($r = -0.25$, $P = 0.004$).

Healthy children were then compared to the collective group of children with neurological disorders (Fig. 3). There was good agreement, except for a higher mean concentration of CCL4 in NIND was undetectable in healthy controls, preventing statistical analysis. When the data were re-analyzed after excluding samples with undetectable levels, no differences were significant after Bonferroni corrections ($P = 0.0029$).

Discussion

This study provides novel observations on a comprehensive panel of chemokines and nonchemokine cytokines in pediatric neurological controls as measured by multiplexed immunoassay. These reference values may be helpful to other investigators studying pediatric neuroinflammation and to clinicians in interpreting published cytokine data in children. They also provide information to assist in selection of cytokines for inclusion in a kit.

TABLE 3. SIGNIFICANT INTERCORRELATIONS OF CYTOKINES

Fluid	Comparison	r	P value
CSF	CCL2 vs. CXCL8	0.56	<0.0001
	IL-6	0.54	<0.0001
Serum	CXCL1 vs. CCL22	0.43	0.0005
	CCL22 vs. IL-12(p40)	0.42	0.0009
	CCL2 vs. GM-CSF	0.61	<0.0001
	CCL11	0.83	<0.0001
	IL-4	0.46	0.0002
	GM-CSF vs. CCL11	0.74	<0.0001
	IL-4	0.90	<0.0001
	CCL11 vs. IL-4	0.69	<0.0001

The required level of statistical significance after Bonferroni corrections was $P < 0.0015$.

GM-CSF, granulocyte-monocyte colony-stimulating factor.

There have been few studies of CSF cytokines in afebrile children, and often with small sample size, whereas febrile children as controls have been better characterized (Asano and others 2010a, b). The control data are often sequestered in publications about a particular disease and reflect evaluation of a limited number of cytokines. In one study of 5 CSF cytokines in 19 neurological controls using a cytometric bead assay from a different vendor, the concentrations of TNF- α , IL-2, IL-6, and IL-4 were in the 2–6 pg/mL range (Aydin and others 2010). In another study utilizing 20 children with non-neurological disorders as controls (Ishizu and others 2006), the CSF concentration of the following cytokines was <1 pg/mL: IL-1 β , IL-2, IL-4, IL-5, IL-7, IL-10, and IL-12(p70). The concentration of these in our study was also trivial. We measured a broader cytokine panel and provide specifics about the frequency of detectable levels and comparison of means and medians.

In serum, which is much more accessible in children, there have been more studies. In one study of 70 healthy older children (≥ 6 years, mean age 12), 8 cytokines were measured by multiplex cytokine assay (Invitrogen) on a Luminex 100 platform. Only sIL2R and IL-12 were found in high concentrations (Bos-Veneman and others 2010), and the median serum IL-2, IL-5, TNF- α , and IFN- γ concentrations were <1 pg/mL. Detection rates were 73% for IL-2, 30% for IL-4, 82% for IL-5, 66% for IFN- γ , and 14% for TNF- α , which showed some differences from our study that utilized kits from a different manufacturer. When multiplex bead-based assays from various vendors were compared for detection of soluble cytokines using the Luminex 100, the conclusion was that kits from the same supplier should be used for serial measurements and when comparisons of absolute values is critical (Khan and others 2004).

Other observations also were made. Chemokines were present in higher concentrations than nonchemokine cytokines in CSF and serum from children. For only 7 or 8 cytokines could both mean and median values be computed. Also, only 6 different cytokines were detectable in 100% of either CSF or serum samples, and 5 of them were chemokines. We conclude that the most reliable and interpretable data would come from the study of those cytokines. CXCL10 and CCL2 were highly detectable in both CSF and serum, whereas IL-6, CXCL8, and IL-1Ra showed much greater detectability in CSF. Serum-preponderant cytokines included CCL5, IL-12(p40), CCL3, and GM-CSF, among others.

The present study addresses the feasibility of using samples from a noninflammatory neurological disorder in studies of pediatric neuroinflammatory disorders. While no comparison could be made for CSF, healthy children and pediatric neurological controls had comparable serum levels of most of the cytokines measured, at least for the types of neurological disorders included. Inclusion of healthy pediatric controls in addition to NIND in studies of serum cytokines in neuroinflammatory disorders is feasible. We are in the process of collecting CSF from noninflammatory, non-neurological disorders for comparison with NIND.

In summary, multiple bead-based immunoassays provide a powerful research tool with the following caveats. First, many of the kit analytes are present in concentrations that are too low for reliable interpretations, have non-computable median values, and a low percentage of samples with detectable levels. As a result, they present challenges to statistical analysis. We recommend reporting these details to

FIG. 3. Comparison of mean serum cytokine concentrations in healthy controls versus neurological controls (NIND). Statistical significance by *t*-tests is indicated. Except for CCL4, nothing remained significant after Bonferroni corrections, or after exclusion of undetectable levels. The two *P*-values for CCL4 are for analysis with and without undetectable values.

readers. Second, the multiplex kits are very expensive, and many of the analytes are not useful for a particular disease or body fluid type. Once it has been determined that a disease does not show a spike in cytokines that are undetectable in controls, a reduced list of user-selected rather than manufacturer-selected analytes is advised for cost-effectiveness and interpretability. Third, for long-term projects, mergers in vendor companies and changes in the antibodies they select may have a significant impact on the cytokine concentrations. What this field needs is absolute standards for cytokines, so results from different laboratories can be compared, but for the foreseeable future, there are going to be differences in antibodies between companies due to the hybridoma process of producing commercial antibodies. Finally, the most sensitive method, whether multiplex bead-based or enzyme-linked immunosorbent assay, should be selected for measuring the cytokines of interest.

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Author Disclosure Statement

All authors have no conflict of interest.

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