Detection of Interleukins 6 and 8, Tumor Necrosis Factor-α, and Soluble Interleukin-2 Receptor in Sera of Healthy Children by an Automated System (Immulite®)

Nachweis von Interleukin-6 und -8, Tumornekrosefaktor-α und löslichem Interleukin-2-Rezeptor mittels eines automatisierten Systems (Immulite®) in Seren gesunder Kinder

U. Sack[1,2], H.-G. Lambrecht[3], Manja Kamprad[1], U. Burkhardt[4], M. Borte[5], F. Emmrich[1]

Summary: Measurement of multiple cytokines requires an automated system for large numbers of samples. We have investigated serum levels of interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and soluble interleukin-2 receptor (sIL-2R; sCD25) in 300 healthy children aged 3 to 17 years using the Immulite® automated immunoassay system. Precision measurement revealed the system to yield highly reproducible results. IL-8 was hardly detectable in healthy children, in contrast to adults. However, mean levels of both TNF-α and sIL-2R were higher in children than in healthy adults. Furthermore, TNF-α levels showed a high inter-individual variability. During early childhood, levels of IL-6 and sIL-2R decline from initially elevated levels. In summary, the Immulite®-system is a reliable, precise, reproducible, and rapid system for cytokine measurement in children. Serum levels of cytokines have been confirmed to be age-dependent.

Keywords: Cytokines/serum; Interleukins/serum; Reference Values; Time Factors.

Zusammenfassung: Für die Messung zahlreicher Zytokine sind automatisierte Analysensysteme mit hohem Probendurchsatz von Vorteil. Wir haben die Serumspiegel von Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumornekrosefaktor-α (TNF-α) und löslichem Interleukin-2-Rezeptor (sIL-2R; sCD25) bei 300 gesunden Kindern zwischen 3 und 17 Jahren mit einem automatisierten Immunoassaysystem (Immulite®) bestimmt. Das System arbeitet mit hoher Präzision. IL-8 konnte bei gesunden Kindern im Gegensatz zu Erwachsenen kaum nachgewiesen werden, während die Spiegel von TNF-α und sIL-2R höher waren. Für TNF-α wurden starke interindividuelle Schwankungen gefunden. Die Konzentrationen von IL-6 und sIL-2R fallen nach initial erhöhten Werten ab. Das Immulite®-System hat sich als ein zuverlässiges, genaues, reproduzierbares und schnelles System für den Zytokin-Nachweis bei Kindern erwiesen. Die Altersabhängigkeit der Serumspiegel von Zytokinen konnte bestätigt werden.

Schlüsselwörter: Zytokine/Serum; Interleukine/Serum; Referenzwerte; Zeitfaktoren.

The detection of cytokines and cytokine receptors has been shown to be of diagnostic relevance in an increasing number of diseases. In specialized diagnostic procedures, the evaluation of cytokine profiles may improve medical care and facilitate therapeutic decisions not only in diseases such as septicemia, tumors, and systemic inflammation [1], but also in postoperative monitoring [2] or after organ transplantation [3]. The diagnostic application of these analytes is dependent upon the availability of automated systems for rapid analysis and on a solid data base of reference values. With the Immulite® system [4], an automated immunoassay system has become available for cytokine measurement. Nevertheless, in the specific case of children, information concerning the suitability of the Immulite® system is still incomplete. Furthermore, data about age-dependent cytokine concentrations in children must still be confirmed [5]. The subject of the present study was therefore the measurement of serum concentrations of diagnostically relevant cytokines and soluble receptors in healthy children aged 3 to 17 years by the Immulite® system. Interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and soluble interleukin-2 receptor (sIL-2R; sCD25) were determined. Because the assessment of serum cytokine levels appears highly sensitive to the length and the modality of sample handling, particular attention was paid to reducing the time between sampling and freezing of the sera to below 20 minutes and to the standardization of the various preanalytic procedures.

[1]Institute of Clinical Immunology and Transfusion Medicine, University of Leipzig
[2]Author for Correspondence: Dr. Ulrich Sack, Institut für Klinische Immunologie und Transfusionsmedizin, Universität Leipzig, Linnéstraße 3, D-04103 Leipzig, Germany. Fax: + 49-341-97 39306; E-mail. sacu@medizin.uni-leipzig.de
[3]DPC Academy, Bad Nauheim, Germany
[4]Department of Anaesthesiology and Intensive Care, University of Leipzig
[5]Hospital for Sick Children, University of Leipzig
Received: June 14, 1999 / Accepted: August 22, 1999

Materials and Methods

Sera were obtained from 300 healthy children undergoing corrective surgery or a medical check-up (see Fig. 1 for age distribution). All children had been free from infectious diseases for at least 4 weeks before examination and were not under the influence of any treatment. C-reactive protein was measured to exclude ongoing inflammation. All children were included in studies approved by the Research Ethics Committee of the University of Leipzig. Written informed consent was obtained from the parents. All patients with unexpectedly high levels for any of the cytokines were reexaminated to exclude onset of disease directly before or immediately after blood sampling.

Blood samples were collected between 8.00 and 9.00 am by venipuncture and centrifuged within 15 minutes. Serum aliquots were stored at –70 °C until analysis. The specimens were thawed immediately before analysis. 30 sera were measured in pairs as both fresh and frozen samples to exclude artifacts.

The analysis was performed with the Immulite® system (DPC Diagnostic Products Corporation, Los Angeles, CA, distribution in Germany: DPC Biermann GmbH, Bad Nauheim) a fully automatic random access chemiluminescence enhanced enzyme immunoassay system [4]. The Immulite® assays for IL-6 [6], IL-8 [7], TNF-α (further information in the package insert, DPC Products Corporation 1997, Los Angeles, CA), and sIL-2R [8] are based on a solid-phase sandwich chemiluminescence enhanced enzyme immunoassay technique. A polystyrene bead coated with murine monoclonal antibodies specific to the molecule to be measured serves as the solid phase. Enzyme-labeled polyclonal (rabbit) antisera for IL-8, TNF-α and sIL-2R and polyclonal (sheep) for IL-6 are used as detection antibodies.

All assays were calibrated against an international standard except sIL-2R for which no international standard is available. The following international standards were used: IL-6: NIBSC 89/548: IL-8: NIBSC 89/520 and TNF-α: NIBSC 87/650. These standards are provided by the National Institute for Biological Standards and Controls (NIBSC), Potters Bar, UK.

The samples were incubated for 30 min (sIL-2R, IL-8) or 60 min (IL-6, TNF-α) at 37 °C with intermittent agitation. Unbound components were removed after 30 min using a patented centrifugal washing technique. Automatically added chemiluminescence substrate (AMPPD, 3-(2'-spirodamantane)-4-methoxy-4-(3'-phophoryloxy) phenyl-1,2-dioxetane) is converted by the bound enzyme during the following 10-min incubation period to an unstable intermediate. The resulting light emission is directly proportional to the concentration of the analyte in the samples.

Non standardized abbreviations: AMPPD, 3-(2'-spirodamantane)-4-methoxy-4-(3'-phophoryloxy) phenyl-1,2-dioxetane; IL-6, interleukin-6; IL-8, interleukin-8 (TNF-α); sIL-2R, soluble interleukin-2 receptor; TNF-α, tumor necrosis factor-α.

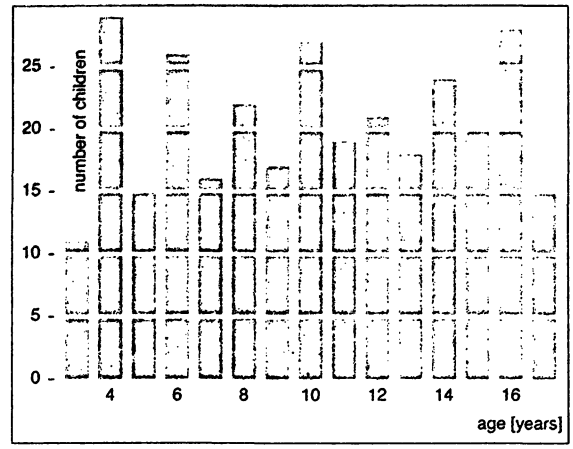

Figure 1 Age distribution of children included in the study.

The stored calibration curve with a 14-day, two-point recalibration interval allows the flexible, economical determination of the immune mediators at any time upon request. To perform measurements of IL-6 at very low concentrations, the lower cut-off was reduced in selected cases.

Precision measurements in the range of the observed analyte concentration was performed with serum pools spiked with recombinant material (IL-6, IL-8, TNF-α from R&D Systems, Minneapolis, MN, U.S.A.; sIL-2R was a gift from DPC) to adjust the concentrations which were expected in the samples. In order to investigate day to day precision, 12 measurements were performed on repeated occasions in independent runs.

Statistical analysis and graphical presentation of data was performed with SigmaStat and SigmaPlot scientific software (SPSS, Chicago, IL, USA), using one way analysis of variance with $\alpha=0.05$ to investigate differences among the age groups, followed by Newman-Keuls test to detect values significantly different to other groups or adults.

Results

Results of inter-assay precision measurement are shown in Tab. 1. At low serum concentrations, coefficients of variation (CV) were up to 34.6% for IL-6 and up to 18.98% for IL-8. In contrast, even at the low concentrations expected in healthy children, CVs were shown to be 5.86% and 4.53%. for TNF-α and sIL-2R, respectively. With the exception of those for TNF-α, CVs decrease with rising serum concentration.

The results of the present analysis of cytokines in sera of healthy children in relationship to age are sum-

Table 1 Results of inter-assay precision measurement. Mean values, standard deviation (SD), and coefficients of variation (CV) are presented as found by repeated measurements on 12 days

IL-6			IL-8			TNF-α			sIL-2R		
Mean (pg/ml)	SD (pg/ml)	CV (%)	mean (pg/ml)	SD (pg/ml)	CV (%)	mean (pg/ml)	SD (pg/ml)	CV (%)	mean (U/ml)	SD (U/ml)	CV (%)
3.68	1.27	34.6	2.99	0.57	18.98	43.1	2.53	5.86	344	15.61	4.53
11.7	1.4	11.9	7.06	0.53	7.51	80.1	6.06	7.57	533	18.96	3.56

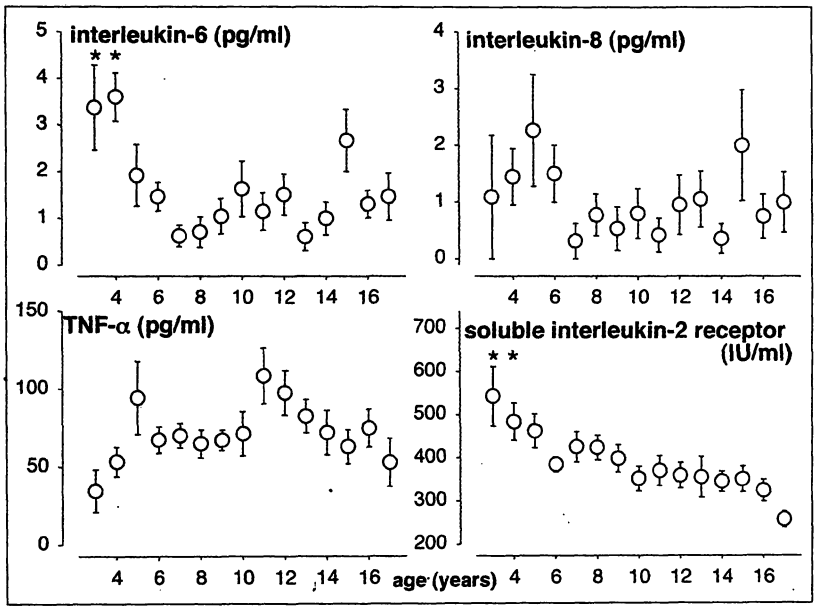

Figure 2 Cytokines in sera of healthy children in relation to age. Points indicating mean values as well as error bars for standard errors of mean (SEM) are shown. Values significantly different ($p<0.05$) as obtained by Newman-Keuls method are indicated by an asterix.

marized in Fig. 2. IL-6 peaked around 3-4 years of age ($p<0.05$ in comparison with later childhood and adult values). IL-8 was detectable only rarely, and in these cases was within the range of normal adults. TNF-α showed overall levels significantly higher than in adults ($p<0.05$). A peak was reached at age 13-14 (not significant in comparison to other ages in childhood), with a rather sharp fall to adult levels thereafter. Soluble IL-2 receptor was significantly enhanced ($p<0.05$) around 3-4 years of age. From age 3 onwards, it presented a progressive decline; however, due to the large variability at very young ages, there was no significant difference with adult levels. There were no statistically significant differences between males and females (data not shown).

In Fig. 3, a comparison between cytokine concentrations measured in fresh and frozen sera is shown. There was no significant difference when freezing was performed within 20 minutes after obtaining the blood sample ($r^2= 0.928$ to 0.989).

Discussion

The present study provides information on the suitability of the Immulite® system for quantification of several cytokines and cytokine receptors of potential diagnostic value in pediatrics. Furthermore, previous findings [5] were confirmed to be independent of the detection system used.

The analytes for the present study were chosen on the basis of their diagnostic potential in children and the availability of an automated system.

Interleukin-6 (IL-6) is one of the most promising candidates for use in routine diagnosis [9, 10]. In healthy children, the serum levels of IL-6 were found to be physiologically elevated around 3-4 years of age, confirming in part previous observations [11]. These findings suggest that IL-6 may play an important physiological role in early childhood. In fact, mRNA for IL-6 has been shown to be expressed in developing cartilage and bone [12]. Although the inter-assay CV of IL-

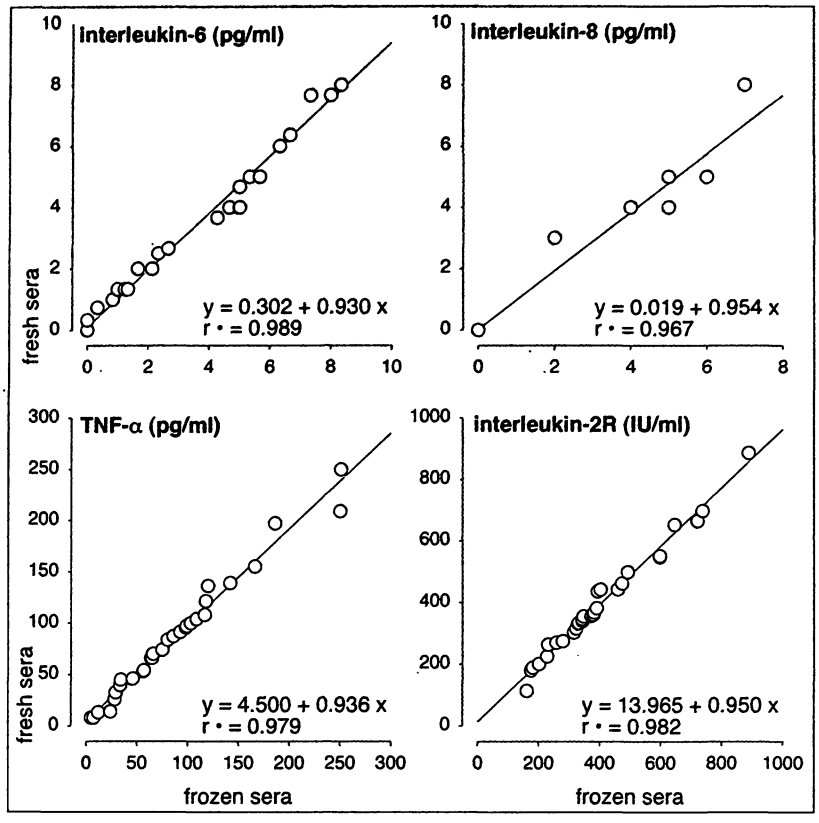

Figure 3 Comparison between cytokine concentrations as measured in fresh and frozen sera. Regression equations and coefficients of correlation are shown.

6 determination has been shown to be rather high at the low concentrations as detected in healthy children, precision improves considerably with concentration.

Interleukin-8 (IL-8), a chemoattractant especially for polymorphonuclear neutrophils [13], had hardly any measurable concentration in the sera of healthy children, indicating that this cytokine is more genuinely associated with inflammation. Similar to IL-6, interassay precision of the Immulite® -System improves with increasing concentration of IL-8.

The concentration of TNF-α was clearly higher in childhood than in adulthood. TNF-α, a multifunctional proinflammatory cytokine [14-18], is remarkably unstable. Therefore, the sera were analyzed very shortly after blood withdrawal, according to a strictly standardized protocol. The results' of this analysis show that TNF-α levels are elevated during the entire period of childhood, but in a profoundly variable fashion among individuals. These observations are highly consistent with similar studies [19] and indicate that TNF-α levels in children must be regarded with great caution, requiring careful adjustments to take account of physiological fluctuation. The precision of the Immulite®-system was excellent at the concentrations found in healthy children.

The sIL-2R (sCD25), released by cleavage of the membrane form of IL-2R [20], can be detected in the sera of patients with immunopathogenetic processes [21, 22]. In contrast to adults, physiologically high concentrations of sIL-2R can be found in the sera of healthy children throughout childhood [23, 24]. sIL-2R showed a progressive decline after age 3, suggesting that physiologically high sIL-2R levels are maintained by the enhanced proliferative activity of the immune system during early childhood, especially in relationship to the thymic maturation of T-cells. With aging and parallel involution of the thymus, sIL-2R declines then to adult levels. The precision of sIL-2R detection with the Immulite®-system is highly satisfactory (CV<5%).

Together, the present findings confirm that cytokine concentrations in the sera of healthy children differ markedly from those of adults. At the low concentration found in these samples, the Immulite®-system enables accurate determinations of cytokine levels. The values detected by this equipment are highly comparable to previously published data [5].

In principle, the detection of immunological mediators by immunoassays can be influenced by the presence of soluble receptors, inhibitors, antagonists, or binding proteins, as well as by degraded or inactive forms of the cytokines. By investigating a large number of healthy children, carefully excluding children with ongoing diseases, and following strict protocols for the rapid and reproducible handling of the speci-

mens, the present study clearly documents elevation of TNF-α. This cytokine can be detected with great precision using the Immulite®-system and appears to be a significant diagnostic indicator..

The levels of IL-6 and sIL-2R differ markedly between the first 3 years of life and the late childhood. Quantitative determinations during this age interval should be performed with careful consideration of appropriate controls. The Immulite®-system provides a reliable tool with which such investigation can be performed in a manner acceptable for laboratory routine diagnostics.

Acknowledgments

Parts of this work were supported by grants from the Saxonian Ministry for Science and Art (SMWK), the Interdisciplinary Centre for Clinical Research at the University of Leipzig (IZKF; grant 01KS9504 A3); the German Research Council (DFG); the Alexander von Humboldt Foundation (Bonn, Germany), and the Sandoz Foundation (Nuremberg, Germany).

The authors would like to thank *Ramona Blaschke*, *Kati Hofmann*, and *Katrin Bauer* for technical assistance, and Dr. *Michael Cross* for critical review of the manuscript.

References

1. Daniel V. Diagnostic value of cytokine determination in serum and plasma. Dtsch Med Wochenschr 1995; 120:1171-4.
2. Tarnok A, Hambsch J, Emmrich F, Sack U, van Son J, Bellinghausen W et al. Complement activation, cytokines, and adhesion molecules in children undergoing cardiac surgery with or without cardiopulmonary bypass. Pediatr Cardiol 1999; 20:113-25.
3. Pirenne J, Pirenne-Noizat F, De Groote D, Vrindts Y, Lopez M, Gathy R et al. Cytokines and organ transplantation. A review. Nucl Med Biol 1994; 21:545-55.
4. Babson AL. The Immulite automated immunoassay system. J Clin Immunoassays 1991;83-8.
5. Sack U, Burkhardt U, Borte M, Schadlich H, Berg K, Emmrich F. Age-dependent levels of select immunological mediators in sera of healthy children. Clin Diagn Lab Immunol 1998; 5:28-32.
6. Klingler W, Schonharl G, Dostatni R, Sustarsic D, Sahakian N, El Shami S. An automated chemiluminescent immunometric assay for interleukin-6 (IL-6) on the Immulite-System. Clin Chem 1996; 42:188.
7. Klingler W, Dostatni R, Pfiztner R, Schonharl G, Sahakian N, Sustarsic D et al. An automated chemiluminescent immunometric assay for interleukin-8 (IL-8) on the Immulite System. Clin Chem 1995; 41:80.
8. Klingler W, Pfiztner R, Schonharl G, Dostatni R, Campbell B, ElShami S. An automated chemiluminescent immunometric assay for interleukin-2 receptor (IL-2R) on the Immulite-System. Clin Chem 1995; 41:53-4.
9. Hirano T, Akira S, Taga T, Kishimoto T. Biological and clinical aspects of interleukin 6. Immunol Today 1990; 11:443-9.
10. Burkhardt U, Sack U, Wild L, Olthoff D. [Serum cytokines and cytokine receptors in children after small or larger surgical interventions with halothane anesthesia]. Anaesthesiol Reanim 1997; 22:50-4.
11. Brailly H, Montero-Julian FA, Zuber CE, Flavetta S, Grassi J, Houssiau F et al. Total interleukin-6 in plasma measured by immunoassay. Clin Chem 1994; 40:116-23.
12. Dodds RA, Merry K, Littlewood A, Gowen M. Expression of mRNA for IL1 beta, IL6 and TGF beta 1 in developing human bone and cartilage. J Histochem Cytochem 1994; 42:733-44.
13. Grob PM, David E, Warren TC, DeLeon RP, Farina PR, Homon CA. Characterization of a receptor for human monocyte-derived neutrophil chemotactic factor/interleukin-8. J Biol Chem 1990; 265:8311-6.
14. Jones EY, Stuart DI, Walker NP. Structure of tumour necrosis factor. Nature 1989; 338:225-8.
15. Smith RA, Baglioni C. The active form of tumor necrosis factor is a trimer. J Biol Chem 1987; 262:6951-4.
16. Beutler B, Cerami A. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu Rev Biochem 1988; 57:505-18.
17. Jacob CO. Tumor necrosis factor alpha in autoimmunity: pretty girl or old witch? Immunol Today 1992; 13:122-5.
18. Tracey KJ, Vlassara H, Cerami A. Cachectin/tumour necrosis factor. Lancet 1989; 1(8647):1122-6.
19. Ishii E, Ohga S, Aoki T, Yamada S, Sako M, Tasaka H et al. Prognosis of children with virus-associated hemophagocytic syndrome and malignant histiocytosis: correlation with levels of serum interleukin-1 and tumor necrosis factor. Acta Haematol 1991; 85:93-9.
20. Rubin LA, Nelson DL. The soluble interleukin-2 receptor: biology, function, and clinical application. Ann Intern Med 1990; 113:619-27.
21. Waldmann TA. The structure, function, and expression of interleukin-2 receptors on normal and malignant lymphocytes. Science 1986; 232:727-32.
22. Adams DH, Wang L, Hubscher SG, Elias E, Neuberger JM. Soluble interleukin-2 receptors in serum and bile of liver transplant recipients. Lancet 1989; 1(8636):469-471.
23. Jones AC, Besley CR, Warner JA, Warner JO. Variations in serum soluble IL-2 receptor concentration. Pediatr Allergy Immunol 1994; 5:230-4.
24. Chan KN, Phillips AD, Walker-Smith JA, MacDonald TT. Serum interleukin-2 receptor in infants and young children. Acta Paediatr 1995; 84:151-5.