

MINIREVIEW

Cytokines and Cytokine Measurements in a Clinical Laboratory

THERESA L. WHITESIDE*

Pittsburgh Cancer Institute and Departments of Pathology and Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

Cytokines are low-molecular-mass (generally, <20,000 kDa) proteins that are produced by leukocytes and a variety of other cell types. Cytokines are increasingly recognized as essential and powerful regulatory and communication molecules. They are a diverse group of proteins involved in homeostasis, defense against infectious agents, and the pathogenesis of many human diseases. Although there are many different cytokines, which are produced by a variety of cell types and which frequently form cytokine networks, several characteristics are universally shared by all cytokines, as follows. (i) Cytokines represent a diverse group of low-molecular-mass proteins with pleiotropic effects; (ii) cytokine production and expression of cytokine mRNA are highly regulated; (iii) cytokines are produced by a variety of cell types; (iv) several types of cytokines may be produced by one cell; (v) cytokine activities may be redundant; (vi) cytokines induce one another and cross-regulate each other's activities; (vii) cytokines bind to cellular receptors, eliciting new mRNA and protein synthesis; and (viii) cytokines are local mediators; the systemic effects of cytokines are quite different from their local effects.

Cytokines are characterized by pleiotropism, or the ability to act upon many different cell types; redundancy, with several cytokines being capable of performing the same function; and a propensity to induce each other, leading to cytokine cascades. Cytokine production occurs in short bursts during the developmental or effector phases of the immune or inflammatory response and is tightly regulated, because of the potential for cytokine-mediated tissue destruction (4). Binding to their cell targets through cell surface receptors, cytokines induce intracellular events which generally lead to new mRNA and protein synthesis, although cytokine-mediated alterations can occur in the absence of protein synthesis. Cytokines may also deliver inhibitory signals. For example, cytokines act as regulators of cell division for certain cell targets, altering the cell cycle and inducing arrest of the G₀-G₁ phase (15). Increasingly, cytokines are being thought of as growth factors or polypeptide regulatory molecules. Their local autocrine or paracrine activities must be contrasted with quite different and often powerful systemic effects. For example, interleukin-1 (IL-1) produced by macrophages locally serves as a costimulatory signal for T-cell activation and proliferation (17), while the consequences of high systemic levels of IL-1 are fever, shock, intravascular coagulation, and leukopenia (10).

On the basis of their main biologic activities, cytokines can be classified into several functional categories. However, as can be seen in Table 1, many cytokines have multiple functions and are listed in more than one of these categories. The prominent

role of the same few cytokines, e.g., in inflammation, immunity, and the host response to infectious agents, indicates that cytokine networks serve the purpose of efficient and rapid mobilization of host defenses when needed. At the same time, these networks need to be regulated, and hence, the network of anti-inflammatory cytokines becomes activated in order to prevent tissue damage and systemic effects, which are associated with high circulating levels of proinflammatory cytokines (Table 1). The realization that cytokines have potent regulatory functions has resulted in a recent profusion of studies designed to elucidate these functions. These studies range from analysis of the molecular regulation of cytokine gene expression through the use of cytokines as therapeutic agents. One example of the therapeutic use of cytokines is for cancer, in which they are being tested as direct antitumor agents, as up-regulators of antitumor immune responses, as potentiators of standard chemo- or radiotherapies, or as agents that are able to decrease the myelotoxic effects of current anticancer treatments (for a review, see references 5 and 6). Another example is the potential clinical use of IL-1 receptor (IL-1R) antagonist (IL-1Ra) or soluble tumor necrosis factor (TNF)-binding proteins to ameliorate the symptoms of septic shock or some immune complex diseases and to counteract the undesirable activity of high levels of proinflammatory cytokines in serum (2, 23). It is believed that blocking of the activity of IL-1 or TNF cannot only reduce but can perhaps completely prevent the detrimental effects of these cytokines (18). However, clinical evaluations of IL-1Ra are still in progress, and its clinical usefulness has not been confirmed.

In view of the rapidly increasing use of cytokines or cytokine antagonists in the therapy of many diseases, assays that measure cytokine levels in sera and/or tissues are becoming an important part of the clinical laboratory repertoire. A variety of approaches to cytokine testing are now available. Bioassays for cytokines have been in use for several years, and although they are sensitive and biologically most relevant, in that they measure only active proteins (Table 2), they are difficult to perform reliably, are time-consuming, and are less specific than immunoassays. The target cells for these bioassays must be maintained in tissue culture, neutralizing antibodies are essential for confirming cytokine identity, and analysis of the S-shaped dose-response curves is not a trivial matter (22). For these reasons, immunoassays for cytokines are generally preferable, and indeed, a variety of enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) kits are now commercially available. A noncompetitive ELISA, which uses a capture (primary) monoclonal antibody, a biotinylated detection (secondary) monoclonal antibody, and streptavidin-enzyme-enzymatic substrate complex, are the most widely used assays (22). These assays are not as sensitive as bioassays but offer specificity, rapid turnaround times, and a relative ease of performance. Competitive EIA, with polyclonal capture anti-

* Mailing address: Pittsburgh Cancer Institute, W1041 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15213. Phone: (412) 624-0096. Fax: (412) 624-0264.

TABLE 1. Functional classification of cytokines^a

Class	Cytokine(s)
Cytokines involved in natural immunity.....	Type I interferons IFN- α and IFN- β , inhibit viral replication, inhibit cell proliferation, activate NK cells, and up-regulate class I MHC molecule expression; TNF- α , mediates host response to gram-negative bacteria and other infectious agents; IL-1- α and - β , mediate host inflammatory response to infectious agents; IL-1Ra, natural antagonist of IL-1 that blocks the signals delivered by IL-1; IL-6, mediates and regulates inflammatory responses; chemokines (IL-8, MCP-1, others), which are involved in leukocyte chemotaxis and activation
Lymphocyte regulatory cytokines	IL-1, costimulates activation of T cells; IL-1Ra, regulates IL-1 activities; IL-2, growth factor for T, B, and NK cells; IL-4, B-cell growth factor; expansion of TH ₂ subset; IL-5, B- and T-cell growth and activation; IL-6, growth factor for B cells; IL-7, stromal cell factor and growth factor for B and T cells; IL-10, inhibits TH ₁ activities; IL-12, expansion of TH ₁ subset, activates effector cells; TNF- β , stimulates effector cell function; TGF- β , antagonizes lymphocytes responses; IFN- γ , activates macrophages and NK cells and up-regulates class I and II MHC molecule expression
Hematopoiesis-regulating cytokines	GM-CSF, G-CSF, M-CSF, colony-stimulating factors; erythropoietin, differentiation of erythroid precursors; IL-2, SCF, <i>c-kit</i> receptor, regulate stem cell development; IL-4, mast cell development; IL-5, eosinophil differentiation and proliferation; IL-6, differentiation of B cells; IL-7, differentiation of B cells
Proinflammatory cytokines	IL-1, TNF- α , IL-6, participate in the acute-phase response and synergize to mediate inflammation, shock, and death
Anti-inflammatory cytokines	IL-4, reduces endotoxin-induced TNF and IL-1 production; IL-6, inhibits TNF production; IL-10, suppresses lymphocyte functions and regulates production of proinflammatory cytokines; TGF- β , has immunosuppressive effects and inhibits IL-1 and TNF gene expression; IL-1Ra, competes with the binding of IL-1 to its cell surface receptors and blocks IL-1R; sTNFR, soluble TNF receptors, by binding TNF, block interaction of TNF with the cell

^a IFN- α and IFN- β , alpha and beta interferons; NK cell, natural killer cell; MHC, major histocompatibility complex; TNF- α , tumor necrosis factor alpha; IL-1, interleukin-1; IL-1Ra, IL-1 receptor antagonist; MCP-1, monocyte chemoattractant protein type 1; TH₁ and TH₂, T-helper and T-helper 2; TGF- β , transforming growth factor β ; GM-CSF, G-CSF, and M-CSF, granulocyte-macrophage-, granulocyte-, and macrophage-colony-stimulating factors, respectively; SCF, stem cell factor.

bodies and biotin-labeled ligands which compete for antibody-binding sites with the ligand in the tested sample, can also be purchased from commercial sources. However, a considerable discrepancy exists between ELISA and competitive EIA. For example, no IL-1 or IL-2 is detectable in normal human serum by ELISA, while nanogram quantities of these cytokines are detectable by EIA (19). The possibility that competitive EIA detects not only free cytokines but also those bound to proteins or soluble cytokine receptors has been considered (19). The biologic significance of such bound versus free cytokines is not clear, although it has been documented that cytokines are capable of binding to, e.g., α 2-macroglobulin in serum (14) and possibly to other serum proteins as well (13).

At the protein level, cytokines may be assayed in serum or other body fluids, including cerebrospinal fluid synovial effusions, bronchial lavages, and peritoneal fluids, as well as in tissues after in vitro extraction of tissue proteins. Measurements of the levels of cytokines in serum may not be informative for a variety of reasons: most cytokines have only short

(minutes) half-lives in serum (4); the frequent presence in sera of soluble cytokine receptors (e.g., sIL-2R, soluble TNF receptors) results in the sequestration of cytokine proteins (5); receptor antagonists, e.g., IL-1Ra, compete for cytokine receptors, blocking IL-1-induced signal transduction, despite the high levels of IL-1 in serum (2, 10); and antibodies to cytokine proteins may be present in the sera of some individuals, particularly those who have received cytokine therapy. In addition, certain cytokines, e.g., TNF- α , may exist and may be active in a membrane-bound or cell-associated form and are not detected unless they are released from the cells, e.g., by freezing and thawing of mononuclear cells (MNCs). Measurements of cytokine levels in serum may be useful in acute inflammation, in persistent, chronic inflammatory states, and for determining the pharmacokinetics of therapeutically administered cytokines.

There is no consensus as to whether plasma or serum should be used for cytokine measurements. Since immune cells are activated during clot formation and cytokines may be released, it is not clear how accurately measurements of cytokine levels in serum reflect the circulating cytokine levels in vivo. In this respect, it may be better to use plasma than serum for cytokine quantitation, provided that the specimens are processed rapidly to avoid the in vitro degradation of cytokines by circulating proteases. Serum samples might contain cytokines secreted from blood leukocytes during the clotting process (8). Because of the possibility of enzymatic digestion leading to cytokine inactivation in vitro, blood or body fluid samples must be processed rapidly and in the cold for cytokine assays. Moreover, samples obtained from patients are often more unstable than those obtained from healthy controls, because higher levels of proteases or other factors which interfere with cytokine assays may be present in pathologic specimens. Methods such as chloroform extraction have been developed; these

TABLE 2. Measurement of cytokines

Assay	Description
Bioassay.....	Very sensitive, only the active form of cytokines is detected; not specific, more than one cytokine can influence functions of indicator cells; neutralization essential to confirm specificity; curve fitting difficult; require tissue culture; results available in days
Immunoassay	Specific but may detect inactive fragments; less sensitive than bioassays; competitive versus noncompetitive immunoassays; easy and available in kit form; results available in hours

methods eliminate the factors in serum that interfere with cytokine determinations (8). Furthermore, the addition of proteinase inhibitors may be considered in those situations in which the proteases in serum interfere with cytokine quantitation (8).

As an alternative to testing cytokine levels in serum, spontaneous or stimulated production of these proteins by MNCs can be measured. Upon *in vitro* stimulation with phytohemagglutinin or lipopolysaccharide, MNCs secrete cytokines, and the levels of these proteins in the supernatant are a reliable measure of an individual's immunocompetence (12). Serial monitoring of individuals over a period of several months has indicated that stimulated cytokine production is a stable trait (21). In addition, spontaneous cytokine release by MNCs indicates that they have been activated *in vivo* (12). While it is recognized that cytokines are produced by a variety of cells, MNCs serve as a convenient vehicle for cytokine assays, mainly because of their easy accessibility. Although the problems associated with testing of serum and the other problems reviewed above are largely eliminated in the MNC system, it requires the use of cells and, as with all indirect procedures, is more time-consuming than serum-based assays. Also, extensive manipulation and cell isolation increase the risk of exposure of laboratory personnel to infectious agents, specifically, to hepatitis viruses and human immunodeficiency virus.

Cytokines are local mediators, and their production can be compartmentalized. Thus, measurement of local cytokine production in tissue or body fluids may be more biologically relevant than that in the peripheral blood. Immunostaining with cytokine-specific monoclonal antibodies can be used to detect these proteins in tissue samples, with a caveat that it may be difficult to distinguish microscopically those cytokines that occupy cellular receptors, and perhaps that are produced elsewhere in tissues, from those that are present in the cytoplasm of the cell. For this reason, it is preferable to measure cytokine gene expression instead of cytokine protein distribution, and a variety of tests are available for this purpose. Assays for cytokine gene expression are generally difficult to perform and interpret, because mRNAs for cytokines are tightly controlled and are rapidly processed; hence, very sensitive assays used at the time when the message is likely to be present are necessary. It has also been suggested that the presence of mRNA does not always mean that a cytokine is produced, because translation may be blocked in certain pathologic situations. In the case of TNF, which is synthesized as a prohormone and which is processed and secreted at a later time, assays of TNF mRNA may not be as informative as assays of TNF protein (1). The newest quantitative technologies for cytokine mRNAs use reverse transcription-competitive PCR (16, 20) and combine the exquisite sensitivity of detection with specificity but, like all of the other RNA-based determinations, are dependent on extraction of RNA from tissues without its degradation. This means that tissues must be snap-frozen immediately after surgery and never allowed to warm up for fear of activating endogenous RNases. While reverse transcription-PCR-based methods may detect the presence of a message for cytokines, they offer no clue about the cellular source or localization of these mRNAs in tissue. *In situ* hybridization for cytokine messages, usually performed with ³⁵S- or digoxigenin-labeled antisense RNA or cDNA probes, offers an opportunity for localization of cytokine mRNAs to specific tissue sites or individual cells (9, 24). While cryosections have been successfully used for *in situ* hybridization to detect cytokine mRNA in human tissues, the possibility of using archival paraffin-embedded tissues has been explored recently with variable and, so far, no truly convincing results

(7). A combination of *in situ* hybridization with PCR, also called *in situ* PCR, has also been evaluated, but with no consistently reliable results for the expression of cytokine genes, as has been the case with a combination of *in situ* hybridization with immunohistology in an attempt to localize the cytokine mRNA to the identifiable individual cells (3, 11). Overall, the methods for cytokine gene expression, although promising, remain in the experimental stage, and cytokine testing in a clinical laboratory relies almost exclusively on immunoassays for cytokine proteins.

Quality control for cytokine assays is essential in a clinical laboratory. Efforts have been made to establish laboratory reference standards for cytokines. These are now available as interim reference standards from the World Health Organization, through the National Institute for Biological Standards and Control in England, or from the Biological Response Modifier Program in Frederick, Md. All cytokine assays should be calibrated against these standards, regardless of assurances provided by or calibrations said to be performed by cytokine kit manufacturers. For the sake of uniformity and convenience, results of cytokine assays should be reported in picograms or nanograms per milliliter instead of arbitrary units. The use of World Health Organization reference standards and recombinant cytokines will facilitate the conversion of immunoassay results into picograms per milliliter. An internal laboratory control for each cytokine (e.g., a supernatant or serum sample with the known level of each cytokine divided into aliquots in vials and stored at -80°C) provides a means for determining interassay variability (a coefficient of variation should not exceed 15%) as well as intraassay variability and should be included in every cytokine assay. Laboratories are obliged to establish their own normal ranges for each cytokine. To detect the possible presence of inhibitors, samples can be spiked with a known amount of authentic cytokine to measure its recovery. These and other quality control issues applicable to cytokines have been reviewed (22).

In view of the enormous progress that is being made in understanding the roles of cytokines in human health and disease, it is likely that cytokine assays of biologic fluids or cells will be performed routinely in hospital laboratories. Several cytokines have been approved for clinical use, and a number of others are in clinical trials alone or in combination with other therapeutic agents for a broad range of diseases. Monitoring of cytokine levels in body fluids, cytokine production by immune cells, or cytokine gene expression by tissue cells are likely to be increasingly used in patients receiving cytokine therapies, particularly in patients with diseases such as cancer or AIDS. The clinical laboratory community should remain sensitive to a growing need for sensitive, accurate, and most importantly, well-controlled cytokine assays.

ACKNOWLEDGMENT

This minireview was supported in part by the Pathology Education and Research Foundation.

REFERENCES

1. Akira, S., T. Hirano, T. Taga, and T. Kishimoto. 1990. Biology of the multifunctional cytokines: IL6 and related molecules (IL1 and TNF). *FASEB J.* 4:2860-2867.
2. Arend, W. P. 1993. IL1 antagonism in inflammatory arthritis. *Lancet* 341:155-156.
3. Bagasra, O., T. Seshamma, and R. J. Pomerantz. 1993. Polymerase chain reaction *in situ*: intracellular amplification and detection of HIV-1 proviral DNA and other specific genes. *J. Immunol. Methods* 158:131-145.
4. Balkwill, F. R., and F. Burke. 1989. The cytokine network. *Immunol. Today* 10:299-304.

5. **Bargetzi, M. J., M. Lantz, C. G. Smith, F. M. Torti, I. Olsson, S. P. Eisenberg, and H. F. Starnes Jr.** 1993. Interleukin 1- β induces interleukin 1 receptor antagonist and tumor necrosis factor binding protein in humans. *Cancer Res.* **53**:4010-4013.
6. **Borden, E. C., and P. M. Sondell.** 1990. Lymphokines and cytokines in cancer treatment. Immunotherapy realized. *Cancer* **65**: 800-814.
7. **Bromley, L., S. McCarthy, J. E. Stickland, C. E. Lewis, and J. O'D. McGee.** Non-isotopic in situ detection of mRNA for interleukin 4 in archival human tissue. *J. Immunol. Methods*, in press.
8. **Cannon, J. G., J. W. van der Meer, D. Kwiatkowski, S. Endres, G. Lonnemann, J. F. Burke, and C. A. Dinarello.** 1988. Interleukin 1-beta in human plasma: optimization of blood collection, plasma extraction, and radioimmunoassay methods. *Lymphokine Res.* **7**:457-467.
9. **Devergne, O., M. Peuchmaur, M. Humbert, E. Navratil, M. B. Leger-Ravet, M. C. Crevon, M. A. Petit, P. Galanaud, and D. Erillie.** 1991. In vivo expression of IL1 beta and IL6 genes during viral infections in humans. *Eur. Cytokine Network* **2**:183-194.
10. **Dinarello, C. A., and S. M. Wolff.** 1993. Mechanisms of disease: the role of interleukin 1 in disease. *N. Engl. J. Med.* **328**:106-113.
11. **Duncan, L. M., L. S. Meehan, and E. R. Unanue.** 1991. IL1 gene expression in lymphoid tissues. *J. Immunol.* **146**:565-571.
12. **Friberg, D., J. Bryant, W. Shannon, and T. L. Whiteside.** 1994. In vitro cytokine production by normal human peripheral blood mononuclear cells as a measure of immunocompetence or the state of activation. *Clin. Diagn. Lab. Immunol.* **1**:261-268.
13. **Hopkins, S. J., and M. Humphreys.** 1990. Bioassay of interleukin 1 in serum and plasma following removal of inhibitory activity with polyethylene glycol. *J. Immunol. Methods* **133**:127-131.
14. **James, K.** 1990. Interactions between cytokines and α 2-macroglobulin. *Immunol. Today* **11**:163-166.
15. **Kimchi, A.** 1993. Cytokine triggered molecular pathways that control cell cycle arrest. *J. Cell. Biochem.* **50**:1-9.
16. **Legoux, P., C. Minty, B. Delpechi, A. J. Minty, and D. Shire.** 1992. Simultaneous quantitation of cytokine mRNAs in interleukin 1 beta stimulated U373 human astrocytoma cells by a polymerization chain reaction method involving co-amplification with internal multispecific control. *Eur. Cytokine Network* **3**:553-563.
17. **Lowenthal, J. W., and H. R. MacDonald.** 1987. Binding and internalization of interleukin 1 by T cells. Direct evidence for high- and low-affinity classes of interleukin 1 receptor. *J. Exp. Med.* **164**:1060-1074.
18. **Ohlsson, K., P. Bjoerk, M. Bergenfeldt, R. Hageman, and R. C. Thompson.** 1990. Interleukin 1 receptor antagonist reduces mortality from endotoxin shock. *Nature (London)* **348**:550-552.
19. **Paciotti, G. F., D. Baron, J. Licinio, L. Tamarkin, W. Mai-Li, P. W. Gold, M. E. Altemus, and D. Rubinow.** 1992. Novel enzyme immunoassays for the detection of the cytokines interleukin 1- α and interleukin 2 in the circulation of normal subjects: 24-hour profiles. *Prog. NeuroEndocrin. Immunol.* **5**:21-30.
20. **Piatak, M., Jr., L. Ka-Cheung, B. Williams, and J. D. Lifson.** 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *Bio/Technology* **14**:70-80.
21. **Rossi, M. I., P. Bonino, T. L. Whiteside, W. B. Flynn, and L. H. Kuller.** 1993. Determination of cytokine production variability in normal individuals over time. *Am. Geriatric Soc. Suppl.* **41**:••• (Abstract.)
22. **Rossio, J. L., H. C. Rager, C. S. Goundry, and E. A. Crisp.** 1992. Cytokine testing in clinical trial monitoring, p. 942-947. *In* N. R. Rose, E. C. DeMascario, J. L. Fahey, H. Friedman, and G. M. Penn (ed.), *Manual of clinical laboratory immunology*, 4th ed. American Society for Microbiology, Washington, D.C.
23. **Spinas, G. A., V. Keller, and M. Brockhaus.** 1992. Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxemia. *J. Clin. Invest.* **90**:533-536.
24. **Vitolo, D., T. Zerbe, A. Kanbour, C. Dahl, R. B. Herberman, and T. L. Whiteside.** 1992. Expression of mRNA for cytokines in tumor-infiltrating mononuclear cells in ovarian adenocarcinoma and invasive breast cancer. *Int. J. Cancer* **51**:573-580.