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Conceptual and Methodological Issues Relevant to Cytokine and Inflammatory Marker Measurements in Clinical Research.

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Abstract

Purpose of review—To provide clinical investigators with an understanding of factors to consider when wishing to add cytokine and inflammatory marker measurements to their studies.

Recent findings—Inflammation involves complex and coordinated responses of the immune system to tissue damage. In the absence of tools to routinely assess inflammation within living tissues, measurements of humoral factors such as cytokines and other inflammatory mediators or markers can provide predictive clinical information plus insights into disease mechanisms. Historically, enzyme-linked immunosorbent assays (ELISAs) became the gold standard, yet this approach of measuring a single protein in each sample limits the amount of information which can be obtained from limited amounts of human sample. In recent years, commercially available multiplex technologies which detect large numbers of proteins in a limited volume have provided investigators with opportunities to begin addressing the complexity of inflammatory responses. Nevertheless, great attention needs to be paid to many aspects of study design, sample collection, sample measurement and data analysis. These considerations are especially significant when using technologies for which experience remains limited.

Summary—While measurements of peripheral levels of inflammatory markers can add important mechanistic elements to human subject research, careful attention to conceptual and methodological considerations is essential, especially when using novel technologies.

Keywords

ELISA; Multiplex; Sample Collection; Sample Handling

Introduction

Inflammation involves a series of coordinated immune responses to tissue damage caused by physical agents (e.g. trauma, surgery or radiation) or pathogens (e.g. bacteria or virus infection) [1]. Cytokines are a diverse group of small secreted soluble proteins which permit communication between immune cells with coordination of inflammatory responses [2].

Inflammation can be classified as either acute or chronic and characterized by tissue or organ redness, pain, heat, swelling and loss of function because of increased local blood supply, capillary permeability and migration of leukocytes out of venules in response to extracellular stimulus [2]. Acute inflammation results from an initial immune response to harmful stimuli resulting in increased movement of plasma, leukocytes, antibodies and complement from the

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blood into injured tissues [1]. When this process fails, evidence of inflammation remains. During chronic inflammation neutrophils are replaced with macrophages and T cells may also be present following infection [1;3]. As a result, destruction of tissue elements may contribute to the development or progression of common autoimmune, cardiovascular and neurodegenerative diseases [3]. In addition to such disease processes, a number of epidemiologic studies of aging have linked elevations in circulating inflammatory markers to the risk of dying, becoming disabled or being frail [4;5].

Recent developments involving highly sensitive Multiplex detection technologies have presented clinical investigators with unprecedented opportunities to measure large numbers of cytokines within a limited sample volume. However, as often happens with new technologies, great initial enthusiasm was soon tempered with important concerns. First, clinical investigators used to dealing with small numbers of commonly used inflammatory markers (e.g. IL-6, TNF-α, CRP), have had to confront the remarkable complexity of designing and analyzing inflammatory marker studies which may potentially include 100 or more different cytokines or chemokines [2]. Second, since currently available Multiplex platforms and more traditional ELISA-based systems exhibit distinct strengths and weaknesses, they should be viewed as complementary tests, with the former being used more for discovery, while the latter may play a confirmatory role [6]. Third, given the current state of Multiplex assays, such measurements cannot simply be contracted out to commercial laboratories [6]. Fourth, none of these discussions preclude the need to carefully consider factors involved in experimental design, sample collection and sample preparation which may profoundly influence study results[6].

The goal of this review is to help clinical investigators design experiments involving inflammatory marker measurements. To that end, major cytokine classes involved in coordination of the inflammatory response will be discussed, issues relevant to study design will be considered, as will advantages and disadvantages of ELISA and Multiplex measurement technologies.

Cytokines and Chemokines in the Inflammatory Response

Cytokines are large soluble proteins which act as immune regulators through interactions with specific membrane receptors [2;7]. As soluble mediators of innate and adaptive immunity, they are often involved in inflammation through their effects on antigen presentation, bone marrow differentiation, cell recruitment, cell activation and adhesion molecule expression.

Source of cytokines and their roles in inflammation

Cytokines are made by many cells, yet inflammatory cytokines listed in Table 1 are mostly produced by CD4+ T helper lymphocytes (Th) (T-lymphocytic derived) and macrophages (mononuclear-phagocytic derived). Among these, type 1 helper (Th1) and type 2 helper (Th2) were characterized by the secretion of distinct cytokines with a variety of functions [8]. Upon encounter with antigen-presenting cells (APCs), naïve CD4+ T cells can differentiate into either interferon-γ (IFN-γ) producing Th1 cells which interact with mononuclear phagocytes destroying intracellular pathogens, or into IL-4 producing Th2 cells which interact with B cells helping them to divide, differentiate and make antibody. Besides IFN-γ and IL-4, Th1 cells also secrete TNF-β and IL-2, while Th2 cells secrete IL-5 and IL-13. While IL-10 was previously classified as a Th2 cytokine, IL-10 is largely derived from regulatory T cells (Treg) and has an anti-inflammatory property to downregulate inflammatory cytokine production including TNF-α, IL-1 and IL-6[9]. Beyond their roles in clearing foreign antigens, abnormal Th1 or Th2 responses may contribute to autoimmune diseases. IL-6 may play a role in the development of T helper 17 (Th17) cells which are the preferential producers of IL-17A,

IL-17F, IL-21, IL-26 and IL-22 [10;11]. IL-17A and IL-17F as a heterodimer signal activate NF-κB driving inflammatory processes [10;11].

Monocytes are able to migrate from blood into tissues where they can differentiate into inflammatory dendritic cells (DCs) or macrophages. DCs regulate the pathogen-specific adaptive immune response and mediate the development of immunologic memory and tolerance or during inflammation [12]. Macrophages remove apoptotic cells, produce growth factors, and phagocytose based on their pathogen recognition receptors [3]. Monocytes, macrophages and DCs release inflammatory cytokines and chemokines [3].

Anti-inflammatory cytokines include IL-4 and IL-10. IL-4 inhibits IFN-γ activation, while IL-10 inhibits synthesis of IL-12, other pro-inflammatory cytokines and the Th17 cytokine IL-17E. The effector function of all T helper cells is regulated by regulatory T cells (Tregs). This T-cell subset defined by Foxp3 and CD25, has a crucial role in controlling allergic disease such as asthma by secreting transforming-growth factor-β (TGF-β) and IL-10.

The roles of Chemokines and Chemokine receptors in inflammation

Chemokines are small chemotactic cytokines which are involved in neutrophil and monocyte cell trafficking [13]. Most chemokines have ability to bind one or more G-protein-coupled receptor (GPCR) to regulate a diversity of signal transduction pathways [13]. Chemokines have only short half-lives and act on cells in close proximity to their release. As a result, few chemokines are present at measurable level in the peripheral blood of most healthy individuals. Even manu pro-inflammatory cytokines are only detectable in the peripheral circulation in response to pathological changes. Nevertheless, the pattern of cytokines/chemokines released or persisting at an inflammatory site may offer important insights into the pathogenesis of many disease processes.

Sample Collection Issues

Reported levels of circulating cytokines can be impacted by sample collection protocols, such as sample handling, processing and storage, and even patient behaviors prior to the collection. Variables such as the time of day that the sample is obtained, the feeding state and acute stress of the patient, the method of storage, duration of storage, and anticoagulant choice can influence the reported levels of circulating cytokines. Standardized procedures for the collection of blood samples and the storage of plasma prior to assay is recommended, so that inter-assay variations are kept to a minimum and meaningful comparable data are produced. For plasma samples, whole blood should be collected in sterile tubes with anticoagulants, placed on ice immediately, and spun down as soon as possible, preferably within 30 minutes. Many cytokines have a short-lived life and begin to degrade once drawn. Thus, small aliquots should be maintained, avoiding repeated freeze-thawing.

Relationship to the time of day

Time of day that blood samples are collected should be synchronized, ideally to the morning to attenuate the influence of circadian patterns and to allow for more meaningful comparisons. Certain cytokines, particularly those that are impacted by glucocorticoids, exhibit distinct diurnal variations which are highly predictable [14–16]. Lipopolysaccharide (LPS)-stimulated whole blood, interferon gamma (IFN-gamma), tumour necrosis factor alpha (TNF-alpha), interleukin 1 (IL-1) and IL-12 production exhibit distinct diurnal rhythms that peak in the early morning [17] and are inversely related to the rhythm of plasma cortisol [17].

Impact of fasting or food ingestion

Patients should be instructed to maintain normal dietary habits and to schedule blood sample collection in the morning following an overnight fast. Feeding status can affect cytokine production and action [18;19]. Chronic food intake patterns (ie. obesity or weight loss) have been shown to affect circulating cytokine levels (TNF-α) [20]. Moreover, acute postprandial measurements can be affected by feeding. For example, circulating C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and IL-6 are elevated following a high-fat meal, while TNF-alpha levels decrease [21;22]. This postprandial effect is likely modulated by the insulin response to acute hyperglycemia [21;23]. Moreover the supplementation of particular antioxidants such as glutathione, vitamins E and C can attenuate the feeding-induced rise in plasma cytokines [23;24]. Hence patients should also be instructed to avoid such supplements prior to testing.

Role of physical activity, exercise and stress

Patients should be instructed to maintain their normal levels of physical activity and to avoid any unconventional strenuous activity prior to blood collection for cytokine measurements. Physical stress, exercise, and trauma all can affect cytokine levels in circulation. While chronic exercise training, improves fitness profiles and results in chronically decreased levels of many circulating cytokines, acute exercise protocols result in short term changes in particular cytokines. While, plasma cytokines are produced by many cell types, muscle cells are a major source during exercise [25;26]. However, these particular responses are highly specific to the exercise protocol and physiological strain (duration, nature, and intensity) [27;28]. For instance, prolonged, endurance type exercise protocols result in acute increases (immediate to 2 h post-exercise) of IL-6, followed by IL-1ra and IL-10 [25;29].

Arterial-venous differences

Blood samples are typically obtained from venipuncture in routine medical care. Venous blood sampling is less invasive than arterial sampling and provides samples after the blood has passed through the tissues. Arterial blood sampling, on the other hand, is rare but occasionally used in specialized procedures since it provides the circulating levels of cytokines prior to the removal by the body tissues. Since cytokines are both removed and produced in local tissues, arterial-venous differences in specific cytokine concentrations are important to consider when comparing cytokine measures. There is also some evidence that local indwelling cannula placement can affect the levels of cytokines (IL-6) as compared to a single needle stick [30]. Thus, artifacts in repeated measures are possible from the local production due to the indwelling cannula rather than actual systemic changes in circulating cytokines.

Serum, plasma or whole blood?

Standard sampling procedures should be implemented when processing blood samples to provide comparable and meaningful values. Cytokine measurement can be affected by the anticoagulant used for the collection of blood and the preparation of biological sample obtained from peripheral blood [31;32]. Serum and plasma are not equivalent. Serum preparation involves the removal of fibrinogen, platelets, and other circulating proteins. This is relevant since cellular elements during the process of coagulation can release inflammatory mediators that may affect cytokine levels [19]. [33] For example, serum samples may contain IL-1 beta secreted from blood leukocytes during the clotting process as compared to EDTA plasma [33]. Peripheral blood collection in sterile EDTA (ethylenediamine tetraacetic acid)-treated tubes has shown to produce the most consistent results for many cytokines [34;35]. Lithium heparin and sodium citrate can decrease the measured levels of some cytokines (IL-6 and TNF-α) as compared to serum or EDTA-plasma [35]. Additionally, lithium heparin in certain

collection tubes containing endotoxin can induce cytokine synthesis [32]. The presence of EDTA can inhibit endotoxin-induced cytokine synthesis [32] and TNF synthesis in the tube [36]. However, EDTA plasma is not recommended for bioassays, since EDTA is a chelating agent. Instead, serum or low-level preservative-free heparin plasma is recommended for bioassays[37;38].

Stability and storage considerations

The short half-life of cytokines, the production of cytokines by cells in peripheral blood preparations, and the potential degradation make the stability, treatment, and storage of biological samples important factors in circulating cytokine measurement, analysis and interpretation [39]. Samples should be collected in sterile tubes, chilled, and processed quickly to maintain cytokine stability [34]. Artifacts in cytokine measurements can be influenced by the length of time plasma or serum is in contact with blood cells [37]. Cytokine production in collected whole blood is apparent in as early as 2-hours following collection [32]. IL-6 levels decrease in whole blood samples left at room temperature (significant after 4 h) [35]. Conversely, TNF-α levels increased, in similar conditions[35]. Thus, plasma or serum should be separated from blood cells quickly to prevent degradation or absorption [38]. Samples should be kept chilled until processing, since differences in cytokine measurements have been observed between those kept at room temperature and −4°C [34]. Samples should be frozen at −80°C until assayed and multiple freeze-thaw cycles should be avoided[38;40]. Most cytokines are stable for up to 2 years of storage at −80°C [40]. However, degradation of IL-13, IL-15, IL-17 and CXCL8 appear within one year of storage, whereas IL-2, IL-4, IL-12 and IL-18 are stable for up to 3 years [40]. Other cytokines, such as IL-1α, IL-1β, IL-5, IL-6, and IL-10 are degraded up to 50% within 2–3 years of storage[40]. After 4 years several cytokines are degraded[40]. For long term storage of samples it may be ideal to use an internal control for multiplex cytokine immunoassays [40]. Most of the cytokines are stable for up to three freeze thaw cycles [34]. However, levels of certain cytokines like TNF-α increase with each successive freeze–thaw cycle, becoming significant after three cycles [35]. Thus, multiple freeze-thaw cycles should be avoided.

Sample preparation in community-based settings

When blood samples are collected in community-based settings, standard operating procedures should be created, formalized, and adhered to. Standards should include detailed patient instructions on preparing for the test, including diet and exercise restrictions, restricting time of day, and sample handing. General guidelines might recommend:

- Blood collection should be done in the morning following an overnight fast

- Patients should be adequately hydrated prior to sampling.

   - It may be appropriate to measure urine specific gravity prior to blood sampling. If hydration is low, patients should consume water until adequate hydration is achieved.

- Physical activity patterns should be maintained in the days preceding the test and uncustomary strenuous activity should be avoided

- Anticoagulants should be appropriately selected based on the desired assay and standardized

- Biological samples should be chilled, quickly processed, and stored at the appropriate temperature and duration to prevent artifacts in the measurement

- Blood samples should be handled appropriately.

- ○ When multiple blood tubes, containing varied anticoagulants are being collected, care must be taken to order tubes appropriately to avoid contamination.

- ○ Required tubes should be pre-chilled prior to collection

- ○ Blood tubes containing EDTA or heparin should be inverted appropriately to allow the anticoagulant to dissolve

- ○ New pipette tips should be used for aliquoting each sample to prevent contamination

ELISA

In the typical double antibody sandwich ELISA, antibody attached to the bottom of a well provides both antigen capture and immune specificity, while another antibody linked to an enzyme provides detection and an amplification factor. This approach enables accurate and sensitive detection of the antigen, the cytokine of interest. As a result, ELISA has been considered the standard cytokine measurement method and commercial ELISA kits are widely used in clinical laboratories and biomedical research[41].

Nevertheless, ELISA performance is highly dependent on antibody quality, kit manufacturer, as well as operator skill and experience [41;42]. In addition, ELISA permits the measurement of only one cytokine at a time in a given sample aliquot, limiting the ability of investigators to measure ever-growing numbers of inflammatory molecules. This concern becomes especially acute when limited amounts of biological material are available. Difficulties also exist in comparing two cytokine levels measured by two different ELISA assays, each under somewhat different conditions.

Another limitation of ELISA-based assays is that the dynamic range (range over which there is a linear relationship between the cytokine concentration and the absorbance reading) is narrow relative to the range for other technologies such as multiplex assays. Thus, samples with cytokine concentrations above the dynamic range have to be diluted for the assay. Dilution not only reduces the concentration of the cytokine being measured, but may also diminish the concentration of any circulating inhibitors or binding proteins. This is particularly relevant to the use of serum samples, exaggerating differences between samples that have cytokine levels within the dynamic range (do not require dilution in the assay) and samples above the dynamic range (do require dilution).

MULTIPLEX ARRAYS

Multiplex arrays measure multiple cytokines in the same sample at the same time [6]. They include flow cytometry, chemiluminescence, or electrochemiluminescence technology, with flow cytometric (bead-based) multiplex assays the most common [6]. Each bead set is coated with a specific capture antibody, and fluorescence or streptavidin-labeled detection antibodies bind to the specific cytokine-capture antibody complex on the bead set. Thus, multiple cytokines in a biological liquid sample can be recognized and measured by the differences in both bead sets, with chromogenic or fluorogenic emissions detected using flow cytometric analysis. Commercially available bead-conjugated antibodies permit the measurement of up to 25 different cytokines in the same sample. However, this number can be greatly expanded if the investigator is willing to custom-conjugate antibodies of interest to one of nearly 100 different available beads.

The advantages of multiplex arrays when compared to ELISA include: a) high throughput multiplex analysis; b) less sample volume needed, c) efficiency in terms of time and cost, d) ability to evaluate the levels of one given inflammatory molecule in the context of multiple

others; e) ability to perform repeated measures of the same cytokine panels in the same subjects under the same experimental assay conditions; f) ability to reliably detect different proteins across a broad dynamic range of concentrations.

Nevertheless, caution is necessary when considering the application of multiplex arrays in clinical research. Experience with multiplex arrays remains limited. Although good correlations between ELISA and multiplex have been reported [43;44], careful side-by-side comparisons to other technologies are rare[45–47]. In addition, while concordance between ELISA and multiplex is generally good when using tissue culture supernatant samples, it is much less robust when using serum or plasma samples [48].

Also since multiplex assays involve potential interactions between multiple different antibodies and cytokines (antigens) in the sample/assay solution, it cannot be assumed that a reliable uniplex assay can just be simply added to a functioning multiplex assay. Non-reactivity to all other antibodies must first be established and the lowest amount possible must be used to minimize such cross-reactions. Problems can also arise from presence of a broad and varying dynamic range with rare and abundant proteins being assayed together.

Abundant circulating proteins in serum or plasma samples, may also affect multiplex results since all bead-based multiplex arrays reactions take place among molecules and antigens which are freely mobile in solution. As a result, these multiplex arrays appear to be much more sensitive than are ELISAs to altered levels of circulating proteins and inhibitors. As many such abundant circulating proteins may change their levels during aging, inflammation or diseases, this can obviously further complicate the picture.

Finally, as in the case of multiple comparison issues when conducting microarray data analysis, multiplex data interpretation can be challenging, requiring careful knowledge of the molecular pathways that lead to cytokine regulation, and careful attention to both study design and data analysis. Moreover, such studies often require the evaluation of dynamic cytokine release by well-characterized lymphocyte subpopulations[49;50].

Conclusion

While measurements of peripheral levels of inflammatory markers can add important mechanistic elements to human subject research, careful attention to conceptual and methodological considerations is essential, especially when using novel technologies. New Multiplex technologies provide opportunities for moving beyond measurements of single molecules to the evaluation of inflammatory pathways and networks. Nevertheless, in spite of many advantages, these new technologies need to be used with caution, with results confirmed using more traditional ELISAs. Furthermore, study design issues usch as the timing and manner in which samples are collected and then processed from study subjects also need to be carefully considered.

Acknowledgments

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Table 1

Cytokine	Cell source	Functions
Proinflammatory Cytokines		
IL-1	Macrophage Dentritic cell	Enhance inflammatory responses Induce fever, sickness behavior Synthesis and release of acute phase proteins
IL-5	Th2 Mast cell	Eosinophil granulocyte proliferation, activation and differentiation, B cell proliferation and secretion Generation of cyotoxic T cell
IL-6	Macrophage Th2 cell Endothelium	Synthesis and release of acute phase proteins Proliferation Inhibitory effect on TNF-α and IL-1
IL-12	Dendritic cell Macrophage, B cell	Th cell differentiaton TNF-α, IFN-γ synthesis
TNF-α	Macrophage Dentritic cell (DC) fibroblast	Stimulate acute phase reaction Cause leukocyte recruitment, vascular leak, mediate septic shock Induce apoptosis Inhibit tumorigenesis and viral replication
IFN-γ	Th1 cell Natural killer cell (NK cell)	Critical role on innate and adaptive immunity against viral and bacterial infection Stimulate macrophages, suppress Th2 cell activity Th1 cell development Promote NK cell activity, leukocyte migration Tumor control
IL-17A	Th17 cell	Promote inflammatory response especially allergic response Mediate autoimmune diseases and transplantation rejection Most cases IL-23 dependent
Anti-inflammatory Cytokines		
IL-4	Th2 cell NK cell	Inhibit IFN-γ synthesis Inhibit Th17 cell Th2 cell development and proliferation Induce B cell class switching to IgE Up-regulate MHC class II production
IL-10	Macrophage Th2 cell	Inhibits Th1 pro-inflammatory cytokine sythesis Inhibit T cell proliferatoin
TGF-β	Macrophage Th cell platelet Endothelial cell Chondrocyte	Block the activation of lymphocytes and monocyte derived phagocytes, regulate proinflammatory cytokines production Suppress G1 cell cycle progression Stimulate expansion of T reg cell