[Frontiers in Bioscience 12, 4682-4695, May 1, 2007]

Cytokine quantitation: technologies and applications

Naresh Sachdeva, Deshratn Asthana

Laboratory for Clinical and Biological Studies, University of Miami-Miller School of Medicine, Miami, Florida 33136

TABLE OF CONTENTS

1. ABSTRACT

Assessment of cytokines in body fluids, cells or tissues provides important information in understanding of disease process and designing treatment strategies. Today, wide range of cytokine assays are available, including; measurement of levels of cytokines (direct) or cytokine soluble receptor levels (indirect) in body fluids or cellular supernatants (immunoassays and cytokine bioassays), measurement of cytokines produced by population of cells (multiparametric flow cytometry, magnetic beads based quantitation of cytokine producing cells, mRNA based assays), measurement of cytokines produced by single cells (Enzyme-linked immunospot (ELISPOT), Intra-cytoplasmic cytokine staining (ICC), mRNA based assays) and detection of cytokines in tissues (immunostaining). Improved understanding of cytokine interactions has led to a consensus that simultaneous assessment of many cytokines in a biological sample provides more comprehensive information rather than assessing a single cytokine. Thus, technologies that measure one cytokine at a time are being gradually replaced by multiplex-type formats (DNA and protein microarrays).

2. INTRODUCTION

Cytokines are low molecular weight proteins produced *de novo* by immune as well as non-immune cells in response to an external stimulus and play a key role in regulating host responses to infectious agents, inflammation and hematopoiesis. They generally (although not always) act over short distances and short time spans and at very low concentrations by binding to specific membrane receptors, the cytokine receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter their behavior (gene expression). Responses to cytokines include, increase or decrease in expression of membrane proteins (including cytokine receptors), proliferation and secretion of effecter molecules (1,2). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). The action of cytokines is pleiotropic i.e., they can act on a variety of cellular targets, and redundant i.e., same biological effect can be mediated by several distinct cytokines. Cytokines induce biological effects in selected microenvironments in the body even at minute quantities

(10^{-10} – 10^{-15} M) but at the same time they are powerful mediators in altering the immune response towards infection or inducing inflammation (3). The biology of cytokines is very complex due to a variety of factors such as short half life, low plasma concentrations, pleiotropy, redundancy, variation in their systemic and local effects and much more still remains to be learned.

On the basis of the type of immune response elicited, cytokines have been classified into Th1, Th2 or Th3 type. The Th1 type of cytokines include interleukin – 12 (IL-12), IL-2, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), with IL-12 playing a central role in eliminating intracellular infections, caused by viruses or parasites, modulation of organ-specific autoimmune diseases or mediating allograft rejection (4,5). Recently IL-23 and IL-27 have been identified to contribute to Th1 immune responses but their mechanisms are different than IL-12 (6). The Th2 type of cytokines include; IL-3, IL-4, IL-5, and IL-13 that are involved in promoting antibody-mediated responses (7). The third class of cytokines, Th3 includes the regulatory mediators such as IL-10 and transforming growth factor beta (TGF-β) which are responsible for maintaining a balance in host microenvironment (8). Some of the recently discovered cytokines, IL-19, IL-20, IL-22, IL-24 and IL-26 have been classified into the family of IL-10-related cytokines (9). Searches for new cytokines are now often conducted at the DNA level, identifying genes similar to known cytokine genes.

After antigen stimulation, Th cells (Th0 type) can develop into Th1 cells that secrete IFN-γ and induce B cells to release antibodies of the immunoglobulin G2 isotype (responsible for phagocyte activation and antibody-dependent cellular cytotoxicity) or Th2 cells that secrete IL-4, IL-5, and IL-13. The action of IFN-γ and IL-4 is antagonistic to each other in that one suppresses the action of the other in the host microenvironment. The cytokines which are involved in causing inflammatory responses are termed pro inflammatory cytokines and include TNF-α, IL-1, IL-8 and recently described IL-15 (10), IL-17 (11) and IL-18 (12). Many cytokines play a very important role in growth of various cell types and their role has been implicated in pathogenesis of tumors and myelomas. These include IL-6, insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), stromal cell-derived factor 1 alpha (SDF-1α), IL-21, B-cell stimulating factor 3 (BSF-3) and fibroblast growth factor (FGF) (13). Other groups of cytokines include interferons and chemokines. Interferons, IFN-α and IFN-β inhibit virus replication in infected cells, while IFN-γ also stimulates MHC expression on antigen-presenting cells. Chemokines attract leukocytes to infection sites and have conserved cysteine residues that allow them to be assigned into four groups. The groups, with representative chemokines, are C-C chemokines (RANTES, MCP-1, MIP-1α, and MIP-1β), C-X-C chemokines (IL-8), C chemokines (lymphotactin), and CXXXC chemokines (fractalkine).

Under normal circumstances cytokines are either at undetectable or at very low levels in body fluids and tissues. Therefore, their presence at elevated levels of expression indicates activation of cytokine pathways associated with disease process or inflammation (14, 15). Currently, cytokines and anti-cytokines find a lot of therapeutic applications in treatment of various infectious diseases, chronic inflammation and cancers (Figure 1). Thus assessment of cytokines in body fluids, tissues or cells provides important information in understanding of disease processes and designing treatment strategies. Today, a wide range of cytokine assays are available, providing an opportunity to evaluate their biological role and establish their therapeutic and diagnostic potential (16). Moreover, improved understanding of cytokine interactions has led to a consensus that simultaneous assessment of many cytokines in a biological sample provides more relevant information rather than assessing a single cytokine. Thus the traditional cytokine technologies that measure one cytokine at a time are being gradually replaced by multiplex-type formats which are sensitive enough to detect multiple analytes even in small quantities of biological samples. The review describes in detail various current and upcoming technologies for quantitation of cytokines and chemokines.

3. TYPES OF CYTOKINE ASSAYS

Depending on the type of information required, the cytokines can be measured by using different techniques.

- Enzyme linked immunosorbant assay (ELISA)
- Radioimmunoassay (RIA)
- Chemiluminescence
- Cytokine bioassays
- Multi-parameteric flow cytometry
- Magnetic beads based quantitation or isolation of cytokine producing cells
- mRNA based assays; Quantitative reverse transcriptase linked – polymerase chain reaction (RT-PCR), Northern blotting, *In situ* hybridization (ISH), RNase protection assays (RPA)
- Intra-cytoplasmic cytokine staining (ICC)
- Enzyme-linked immunospot (ELISPOT)
- Immunostaining; Immunofluorescence, Immunocytochemistry
- Cytokine Microarrays; DNA microarrays, Protein microarrays

3.1. Measurement of cytokines (direct) or soluble cytokine receptors (indirect) in the biological fluids or cellular supernatants

3.1.1. Enzyme linked immunosorbant assay (ELISA)

Due to the amplifying potential of enzyme labels, immunoassays that use enzyme-conjugated antibodies have become increasingly popular because of their high specificity, sensitivity, rapid turnaround time, convenience, the ease of performance and relatively low costs. The same holds true for measuring cytokines, which are generally measured by a sandwich ELISA. The basic cytokine sandwich ELISA based method makes use of highly-purified anti-cytokine antibodies (capture antibodies) which are non-covalently adsorbed (“coated” – primarily as

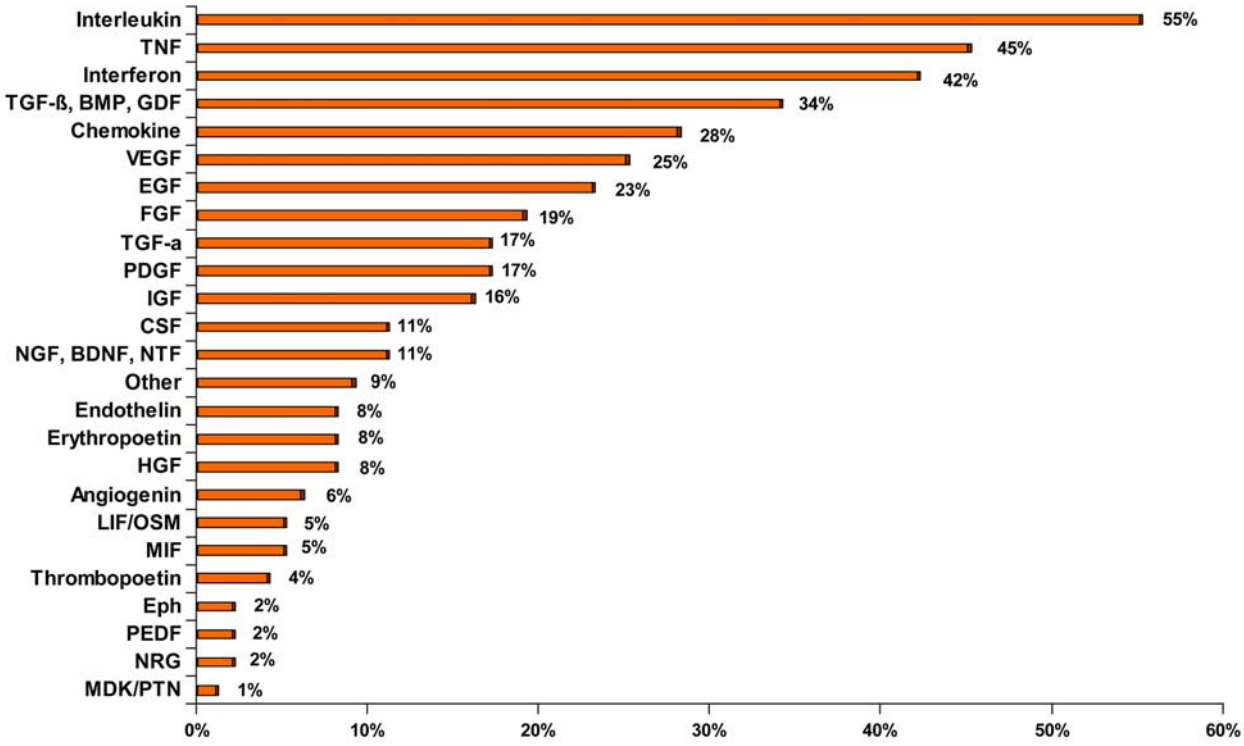

Figure 1. Cytokines comprise of most frequently studied molecules in medical bioscience. Source: The Science advisory board, Arlington, VA, United States (http://www.scienceboard.net).

result of hydrophobic interactions) onto plastic microwell plates. The immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by addition of an enzyme-labeled avidin or streptavidin. After the addition of a chromogenic substrate, the level of colored product generated by the bound enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA-plate reader at an appropriate wavelength. Data storage and reanalysis are greatly simplified when the plate reader is connected to a computer. However, the source and quality of primary antibodies used for capture and detection of cytokines are by far the most important factors for the success of an immunoassay. Other factors that influence cytokine measurements include, detection of biologically inactive cytokines and their fragments, non specific binding of capture antibodies with various other proteins present in the biological fluids (matrix effects) (17). Certain antibodies can detect soluble (secreted) as well as membrane bound forms of the same cytokine, while others detect only soluble cytokine. For example, IL-1β is secreted as a 17-kDa fragment, and it also exists as a larger, 35-kDa molecule (18). Some cytokines can exist under multiple variable forms: monomers/polymers, precursors, various degrees of glycosylation and degradation products which behave differently in immunoassays. Existence of auto-antibodies to cytokines such as those described in case of TNF-α, IL-6 and IL-1 also influence their measurements (19,20). Some of the cytokine inhibitors such as IL-1ra (which binds to the specific IL-1 receptor without signal transduction) also influence measurement of cytokine receptors.

Today there are number of companies that market ELISA kits for a variety of cytokines. Their detection limit is around 1-5 pg/ml and they have good reproducibility (C.V., 5-10%). Unfortunately, the scientific information provided by the manufacturers is generally insufficient to appreciate the analytical performance of the technique. Therefore it is generally recommended to run healthy control samples while analyzing a particular cytokine in biological fluids such as human sera. Although cytokine ELISA is very useful for cytokine detection and measurement, several limitations for the interpretation of ELISA data must be mentioned. For example, test samples often come from tissue culture supernatants or biological fluids which are conditioned with cytokines produced by mixed cell populations; the ELISA data does not provide direct information on the identities and frequencies of individual cytokine producing cells. Techniques such as ELISPOT, Immunofluorescene, ICC or mRNA based assays are recommended for the latter type of analysis (21). Another consideration is that since cytokine concentrations are measured at only one time point, the results do not reflect the concurrent processes of cytokine secretion, cytokine uptake by cells and cytokine protein degradation. Hence, cytokine ELISA is a useful indicator of the presence and levels of cytokines in the biological fluids but it does

not actually provide information concerning the biological potency of the detected proteins.

3.1.2. Radioimmunoassay (RIA)

RIA is highly sensitive and accurate in quantitation of cytokines in biological samples. Like any other immunoassay, RIA or immunoradiometric assays (IRMA) require labeled cytokine-specific antibodies and/or labeled cytokines or their receptors with a radio-isotope (mostly ^{125}I) followed by detection. Micro-plate based RIA can be counted quickly and easily on the beta scintillation counters while assays set up in test tubes can be counted on the gamma counters (22).

Another type of RIA, the radio-receptor assays (RRA) measure concentrations of cytokines by displacing ligands from cell-bound receptors (23). The method was initially developed for identification and quantitation of specific hormone receptors, but is equally good for cytokine measurements. In this assay mixture of the test sample and a known amount of the radio labeled substance under test is exposed to a measured quantity of receptors for the substance and the amount in the test sample is determined from the proportion of receptors occupied by radio labeled molecules of the substance under the assumption that labeled and unlabeled molecules bind to the receptor sites at random. A particular advantage of the RRA is that it has specificity directed towards the biologically active regions of the cytokine, rather than to the immunologically active region that may have little or no involvement in the expression of cytokine activity. However, the assays involving the use of radioisotopes have largely been replaced by non radioactive assays because of considerations of exposure to radiations, labor intensive time consuming procedures and costly equipment requirements such as beta or gamma counters.

3.1.3. Chemiluminescence

The sensitivity of ELISA often depends on the detection limit of the colorimetric substrates used. Chemiluminescence is another dimension to ELISA like immunoassays that involves the cleavage or fragmentation of the O-O bond an organic peroxide compound (substrate) with a peroxidase (enzyme) during the detection of the antigen (cytokine or its receptor). The difference in the properties of substrates in ELISA and chemiluminescence makes the latter technique highly sensitive. For example, the commercially available chemiluminescent peroxidase substrate luminol, is approximately 10 times more sensitive than the commonly used ELISA substrate tetra methyl benzidine (TMB) even though they are catalyzed by the same enzyme horseradish peroxidase (HRP) (24). However, despite of higher sensitivities, the chemiluminescent assays have not been able to replace ELISA based assays due to their high costs.

3.1.4. Cytokine bioassays

As biologically active entities, cytokines cannot be fully characterized by physicochemical methods alone. Thus, biological assays (bioassays) have become increasingly important for their biological characterization and potency determinations. These bioassays include measurement of stimulation or inhibition of cell proliferation, cytotoxicity/apoptosis, antiviral activity, differentiation, and up-regulation of expression of intracellular, secreted, and surface membrane proteins (Table 1) (25). Several cytokines, especially those acting on host defense systems, have been or are being developed as bio-therapeutic agents (e.g., IL-2, GM-CSF, IFN-α, IFN-β) (26-29). Therefore, accurate measurement of cytokine activities has thus become increasingly important (30). In practice, bioassays are more time-consuming and labor-intensive than immunoassays. They require tissue culture facility, because of the requirement for a target cell line, which serves as a read-out of the biological response. Target cell lines used in cytokine bioassays are carefully selected for sensitivity to or dependence on a given cytokine. These cell lines are generally available commercially or from the American Type Culture Collection (ATCC), a non-profit organization in Manassas, VA, which serves as a repository for cell lines of various origins (31). Bioassays conducted in different laboratories often depend on variable sources of somatic cells/cell lines and culture reagents, and are subject to variation in both design and methodological details. Thus, there are usually no 'reference' bioassays to measure potency. Therefore, to enable accurate calibration of potency estimates (determinations), it is essential that bioassays are monitored for sensitivity on an 'assay-to-assay' basis. This is best done, with the inclusion of appropriate, well-characterized, biological standards. Priorities for cytokine standardization are set by the World Health Organization's (WHO) consultative group on standards of cytokines, growth factors and endocrinological substances, composed of experts in these fields, which meet every 2–3 years in collaboration with the Center for Biologics Evaluation and Research (The National Institutes of Health (NIH), Bethesda, MD, USA), (7th meeting held at National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK on 20–21 October 2003) (32,33). Final adoption and establishment of WHO international standards and reference ranges is decided by WHO's Expert Committee on Biological Standardization (ECBS).

Based on the type of function measured in the target cells incubated in the presence of a cytokine, the bioassays may be divided into five broad categories: (i) assays which measure proliferation or growth inhibition of target cell lines; (ii) assays measuring cytotoxicity; (iii) assays dependent on the induction of a specific cell function, such as chemotaxis; (iv) assays estimating the quantity of a protein induced in the target cell and (v) assays estimating the killing of intracellular pathogens such as viruses. In cytokine-driven proliferation assays, growth of target cells is the endpoint: the cells stop growing unless the relevant cytokine is present. A good example is a response of B9 cells to IL-6, as described by Remick (34). These cells will not only increase in number in the presence of IL-6, but in the absence of IL-6, they will die out over time. Another common bioassay utilizes CTLL-2 cells for measuring the presence of IL-2 (35). Other bioassays depend on the ability of cytokines to induce death of target cells, as in a bioassay for tumor necrosis factor (36). The number of cells are counted at the end of the assay by incorporation of tritiated thymidine, manual cell counts, or

Table 1. Bioassays for measurement of potency of some of the commonly investigated cytokines

Cytokine	Suitable cell line	Functional response	Overlapping/modulating cytokines
GM-CSF	MO7e, human megakaryoblastic leukemia, or TF-1, human erythroleukemia	Proliferation	IL-4, -5, -6, -13, -15, GM-CSF, NGF, SCF, LIF, EPO, OSM, TNFα/β, IFN-α/β, TGF-β
IFN-α	A549, human lung carcinoma, or 2D9, human glioblastoma, or Daudi, Burkitts lymphoma, or Transfected A549 or HEK 293 (human embryo kidney) cells	Antiviral activity, or production of protein MxA (by ELISA)	IFN-β, IFN-x, IFN-c
IFN-γ	A549, human lung carcinoma, or 2D9, human glioblastoma, or Colo205, human colon adenocarcinoma	Antiviral activity	IFN-α, IFN-β
IL-2	CTLL-2, murine cytotoxic T cell	Proliferation	IL-12, IL-15, TGF-β1, TGF-β2
IL-4	CT.h4S, murine cytotoxic T cell transfected with human IL-4 receptor	Proliferation	IL-2, TNF-α/β, IFN-α/β, TGF-β
IL-6	B9, murine hybridoma	Proliferation	IL-11, IL-13, OSM
IL-10	Ba8.1C1, murine pro-B cell transfected with human IL-10 receptor	Proliferation	-
IL-12	KIT-225, human chronic T-lymphocyte leukemia	Proliferation	IL-2, IL-4, IL-7, IL-15, IL-21, IL-23
IL-18	KG-1, human myelomonocytic leukemia	IFN-c production (by ELISA)	IL-12, IL-21
TNF-α	L-929, murine fibroblast or WEHI164 clone 13, murine fibroblast, or KYM-1, human rhabdomyosarcoma, or U-138MG, human glioblastoma	Cytotoxic activity, Stimulation of ICAM-1 expression	IL-1α, IL-1β, IFN-c, TNF-β

EPO: Erythropoetin, GM-CSF: Granulocyte macrophage colony stimulating factor, IFN- Interferon, LIF: Leukemia inhibitory factor, NGF: Nerve growth factor, OSM: Oncostatin M, SCF: Stem cell factor, TGF: Transforming growth factor, TNF: Tumor necrosis factor. Ref (25).

by non-radioactive methods, e.g., those based on the use of MTT tetrazolium (3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide) (37,38), water-soluble tetrazolium salts or by flow cytometry (incorporation of carboxy fluoroscein succinimidyl ester; CFSE stain). Certain cytokines, such as TNF-α or TNF-β, exert a direct cytotoxic effect on susceptible target cells (39,40). In cytotoxicity assays, the number of target cells killed during the assay are measured and related to the concentration of the cytokine in the biological fluid. Alternatively, the uptake of a dye (e.g., naphthol blue-black or neutral red) by residual viable cells at the end of the assay can be quantified, visually on a microscope or using an ELISA reader. An example of this response is the WEHI 164 cell line, which is very sensitive to the toxic effects of TNF (34).

The third category of cytokine bioassays involves measurement of their functions other than proliferation and killing. These include; secretion of secondary cytokines (41), changes in phosphorylation of signaling molecules (42), alterations in the calcium flux (43), up-regulation of the major MHC molecules (44), release of cytoplasmic granules (45), induction of respiratory burst in macrophages or granulocytes (46) or promotion of chemotaxis by the chemokines (47-49). The fourth type of bioassay depends on the ability of a cytokine to induce the production of a protein in the target cell. One such example is a bioassay for IL-18, which depends on the ability of IL-18 to induce IFN-γ secretion by T lymphocytes (50,51). Antiviral assays are included in the fifth category (52,53). These involve the preincubation of susceptible cells with cytokines, such as interferons followed by measurements of an increased survival and protection from cytopathic effects of a virus challenge, such as encephalomyocarditis virus or vesicular stomatitis virus. Target cells are human primary fibroblasts or cultured Hep 2C, WISH, or A549 cell lines, and the number of viable target cells are determined at the end of the assay. Some assays measure induction of target genes, such as MxA gene induction assay in case of determination of neutralizing antibodies to IFN-β1b, in multiple sclerosis patients treated with IFN-β (54).

Even though bioassays are infrequently performed because of issues related to time and labor, they are gold standards for testing of cytokines since they measure their bioactivity and not just presence or levels. Thus bioassays are often used for confirmation and validation of results obtained in other cytokine assays. The other pitfalls associated with cytokine bioassays include, lack of specificity, since the target cells respond to multiple growth factors and chemicals in the biological settings; presence of inhibitors (e.g. IL-1β antagonists) or antibodies (51,55); non specific inhibitors (albumins, lipids, α-2 macroglobulin) (56,57) etc.

3.2. Measurement of cytokines produced by population of cells, (spontaneous or stimulated)

3.2.1. Multi-parametric flow cytometry

Flow cytometry is a simple and powerful method in which the individual cells can be simultaneously analyzed for many parameters including intracellular expression of cytokines (described later) by using fluorescent labeled antibodies. The recent availability of particle based flow cytometric assays have made multiple quantitation of cytokines secreted in plasma or other biological fluids possible (58,59). The technology utilizes micro spheres or beads as the solid support for a conventional immunoassay, which are subsequently analyzed on a flow cytometer (Figure 2). The combined advantages of the broad dynamic range of fluorescence detection via flow cytometry and the efficient capturing of analytes via suspended particles enables one to use fewer sample dilutions and measure the cytokine concentrations in substantially less time compared to conventional ELISA. Using different combinations of the fluorochrome conjugated antibodies one can also obtain information on simultaneous production of distinct cytokines by a single cell type such as CD4/CD8 T cells or NK cells (60).

Several multiplexed bead systems are currently marketed by different vendors for simultaneous detection of multiple cytokines. For example, a multiplexed particle based flow cytometric assay available from Luminex

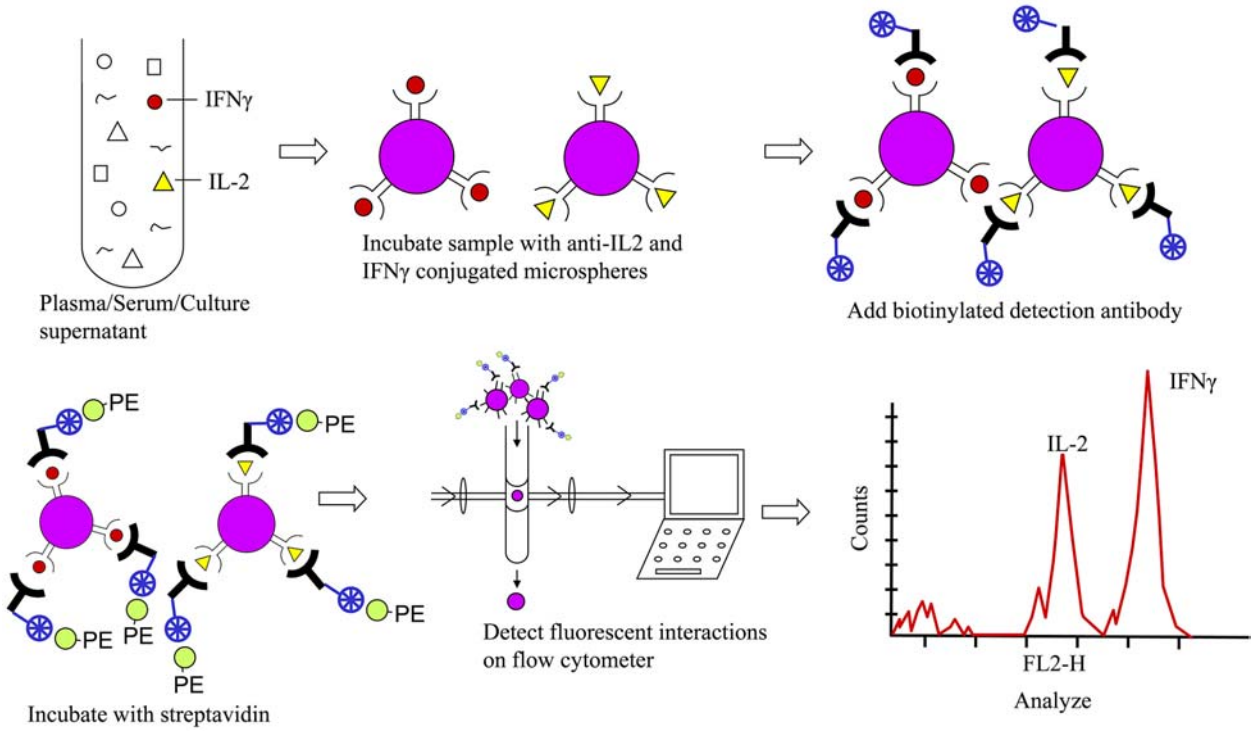

Figure 2. Flow cytometric detection of cytokines in biological specimens.

Flowmetrix system which consists of different bead sets manufactured with uniform, distinct proportions of red and orange fluorescent dyes, can simultaneously measure up to 25 cytokines and chemokines in a single sample (61,62). Similarly, the BD Biosciences offer a cytometric bead array capable of distinguishing Th1 and Th2 cytokines and detection of pro- inflammatory cytokines in a given sample (63).

3.2.2. Magnetic beads based quantitation or isolation of cytokine producing cells

Magnetic bead technology allows simple, rapid and efficient enrichment or depletion of specific cell populations. The monoclonal antibody-labeled magnetic particles (size 0.1-0.45 μm) provide ultimate flexibility for enrichment of cytokine producing leukocyte subpopulations as well. The cytokine producing cell populations are isolated in presence of magnetic field (applied by magnetic columns), after which they can be analyzed for production of cytokines by single cell assays (described later) (64). McNeill *et al* (65) have recently developed a magnetic polymer cytokine immunoassay which utilizes magnetic polymer beads to detect cytokines, using standard 96-well plates in an ELISA based format. The technique is as sensitive as sandwich ELISA and does not require any flow cytometry equipment. Magnetic bead based cell separation offers a cost effective alternative for cell separation as compared to flow cytometric cell sorting. However, since the separation of cells is carried out in the presence of strong magnetic fields, the technique may influence cytokine production by the separated cells.

3.2.3. mRNA based assays

Methods for detection of cytokine transcripts in cells or tissues generally employ reverse transcription PCR (RT-PCR), RNase protection assays (RPA), or in situ hybridization (ISH). Of the three methods for cytokine transcript detection, RT-PCR is the most common, although it only identifies the presence of cytokine transcripts but not the cells containing the transcripts (66-68). Its newer version, real-time quantitative PCR, allows measurements of the copy number of a particular transcript relative to the expression level of a housekeeping gene or an endogenous reference, such as ribosomal RNA (rRNA) (69,70). This method enables the investigator to quantitatively compare cytokine gene expression between several different targets. Recently, a real time RT-PCR technique termed CyProQuant-PCR (Cytokine profiling quantitative PCR) was introduced, that uses external RNA standards for profiling of human cytokines (71). The technique is useful for immuno-monitoring of *in-vivo/in-vitro* stimulated cells and powerful enough to precociously detect slight cytokine induction. Micro-plate based assays that measure cytokine-specific mRNA are now also available commercially (72,73). These assays quantitate mRNA from cell lysates in an ELISA format by using gene-specific biotin-labeled capture probes and digoxigenin-conjugated detection probes. The cytokine RNA/probe hybrids are captured on a streptavidin-coated micro-plate and following washing and addition of the substrate (alkaline phosphatase conjugated to anti-digoxigenin antibody), the color is developed and read in a spectrophotometer.

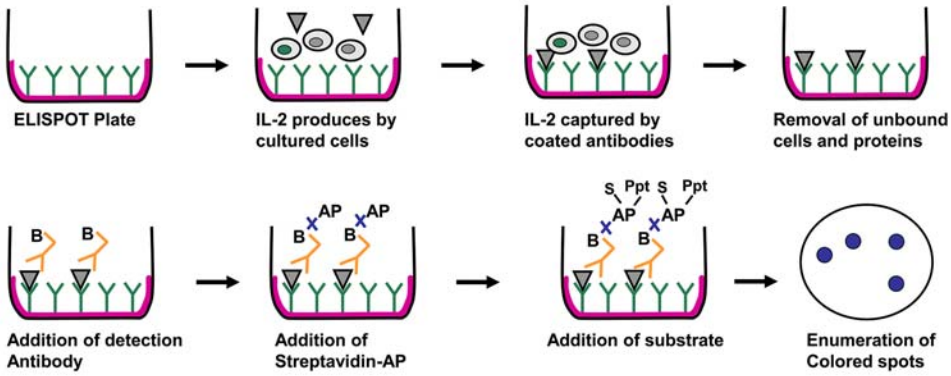

Figure 3. Detection of cytokines (eg: IL-2) released by cells using ELISPOT technique. Membranes at the base of the wells of microplate are coated with anti IL-2. Cells are added to the plate with or without the stimulant. Upon culture, the cells release IL-2 which is captured by the anti IL-2 antibodies coated on the plate. Cells and non-bound proteins are washed away and biotinylated anti-human IL-2 detection antibody is added which binds to IL-2. Streptavidin-AP is added, which binds to the biotinylated detection antibody. A precipitating substrate (NBT/BCIP) is added to the wells which react with AP to produced colored spots, which can be read manually or on a ELISPOT reader. Abbreviations: AP, Alkaline phosphatse; NBT, Nitro blue tetrazolium; BCIP, 5-Bromo-4-choloro-3-indolyl phosphate.

The RPA system is a highly sensitive and specific method for simultaneous detection of up to 12 different mRNA species in a single sample of total RNA (74,75). The method utilizes specific riboprobes, which are generated using DNA-dependent RNA polymerases from the bacteriophages T7 or T3. The resulting linear probes are hybridized with target mRNA. After free probes and other single-stranded RNA molecules are digested with RNase, the hybridized probe/target RNA duplexes are resolved according to size on polyacrylamide gels and transferred to a nylon membrane. Quantitation of the RNA fixed to the membrane by UV cross linking follows, based on the label incorporated into antisense RNA probes (either ^{32}P or biotin-^{16}UTP).

The ISH for cytokine transcripts is less popular than real-time PCR and perhaps more difficult to perform. Its main advantage is that it identifies cytokine transcripts within cells which produce them, rather than their quantitation (76). Northern blotting too, which is conventionally used for long time to detect protein expression, can be used for detection of cytokine transcripts.

3.3. Measurement of cytokines produced by single cells (single cell assays)

3.3.1. Intra-cytoplasmic cytokine staining (ICC)

Cytokine detection in single cells is possible because of recent advances made in flow cytometry. Cell permeabilization is necessary for detection of intra-cytoplasmic, as opposed to cell-surface associated, cytokines. Detergent-based permeabilization methods such as saponin are routinely used for intracellular cytokine detection (77,78). Successful staining for intracellular cytokines requires the use of (i) a protein transport inhibitor, such as brefeldin A or monensin; (ii) appropriate gentle fixation and permeabilzation of cells; (iii) anti-cytokine antibodies that bind to fixed cytokine proteins. Since the procedure demands fixation and permeabilization of cells, these treatments can lead to some artifacts. Fixation of cells may cause non-specific trapping of antibodies leading to increased auto-fluorescence and the permeabilization increases the amount of protein available for non-specific interaction that can be avoided by careful titration of staining antibody. Also, ICC relies on measurement of cytokines at single time point and the sequential expression of different cytokines by the same cells may not be possible. Moreover, ICC has limitations for assessment of cytokines produced in smaller quantities in particular, from distinguishing the cells that produce cytokines form those cells which internalize the cytokines (79). Though the technique is costly, it has an advantage over ELISPOT in providing information on multiple cytokines produced per cell in less amount of time (approximately 6 hours). This method has been widely used in determinations of the frequency of antigen reactive, cytokine-producing T cells in the peripheral circulation or in-vitro stimulated populations of hematopoietic cells using flow cytometry by simultaneous assessment of cell surface markers and cytokines (80). The ICC has also been recommended by the HIV Vaccine Trial Network (HVTN) as one of the flow cytometric methods to monitor immune responses in HIV vaccine trials (81).

3.3.2. Enzyme-linked immunospot (ELISPOT)

The most widely used assay for the assessment of cytokines produced by a single cell is the ELISPOT, which has been developed to determine the frequency of precursor cells in fresh or cultured populations of lymphocytes capable of releasing cytokines in response to stimulation by a cognate antigen (82). The ELISPOT has become an assay of choice for monitoring responses to antitumor and antiviral vaccines for several reasons. Like ELISA, It is performed in 96-well plates bottomed with membranes like polyvinylidene difluoride or nitrocellulose that supports the growth of cells and has the retaining capacity to capture antibodies (Figure 3). The technique is adaptable to a high throughput, can be set up in several different formats either

with unseparated PBMCs or enriched subpopulations of lymphocytes, and results (i.e., the number of spots per well) can be acquired in an unbiased way by image analysis. It also provides an estimate of the frequency of antigen-responsive cells among those plated (83,84). The assay can detect one cytokine producing cell per 10^5 plated making it more sensitive than ICC, and can be performed with cryopreserved PBMCs (85,86). The ELISPOT assays for IFN-g, IL-5, IL- 10 and other cellular products, such as granzyme B, have been useful in the assessment of cytotoxic T lymphocytes (CTLs) precursor frequencies prior and after peptide-based vaccinations in patients with cancer and also in HIV vaccine trials (85,87,88).

Number of variations of the ELISPOT assays has been developed by investigators, such as the dual-color ELISPOT assay capable of differentiating three subtypes of Th cells simultaneously (89). In this system, IL-2 secreting cells (Th1) are developed with Horse radish peroxidase and amino-ethyl-carbazole (produce red spots); IL-4 secreting cells (Th2) with alkaline phosphatase and vector blue (produce blue spots). The Th0 cells that produce both types of cytokines are identified by presence of mixed color indigo spots, as they utilize both the chromogenic substrates. However, the assay has limitations because of the difficulties in the interpretation of mixed color spots (90,91). Multiplex systems have been developed for ELISPOT assays as well. For example, using four different capture antibodies (hIFN-γ, hIL-2, hIL-4, hTNF-α) in combination, Palzer *et al* (92) demonstrated simultaneous detection of 4 cytokines per well. In another modification of the technique, Gazagne *et al* (93) have developed a Fluorospot assay, based on the use of multiple anti-cytokine detection antibodies and a special ELISPOT reader consisting of a light microscope with incident fluorescence illumination. This assay provides slightly better discrimination and characterization of dual cytokine-producing cells than the enzymatic reaction.

3.4. Detection of cytokines in tissues

3.4.1. Immunostaining

Detection and measurement of cytokines in tissues is more relevant since *in situ* cytokine profile provides more information about the ongoing physiologic or pathologic processes than systemic cytokine levels. Methods for *in situ* studies of cytokines can target mRNA or proteins using various labeled probes. Immunohistochemistry based cytokine detection is an antibody-based method (94,95). Depending on the label conjugated to the antibody i.e., an enzyme or a fluorochrome (Immunofluorescence), light or fluorescent microscope is used for cytokine detection. Antibody selection is a critical step in immunostaining procedures, because antibodies that perform admirably in western blots or immunoprecipitation for cytokine proteins may not be suitable for tissue staining. Immunostaining of tissue sections or cells has to be preceded by fixation, to ensure that the cytokines are not lost during the washing procedure. However, even mild fixation might alter the conformation of a cytokine or cause its partial denaturation (96). Such altered cytokines will not be recognized by antibodies made to the cytokine in its native conformation. Therefore, antibodies selected for immunostaining must be able to recognize and bind to a cytokine after fixation. A wide range of polyclonal or monoclonal antibodies to human cytokines are commercially available these days, and it is up to the user to select those that will perform optimally. Nonspecific staining has to be carefully controlled by using isotype controls. Overall, with considerable experience and successful implementation, immunostaining can provide useful information about localization of cells expressing a cytokine and the relative frequency of these cells in tissues (97,98).

3.4.2. mRNA based assays for tissues

The mRNA based assays for tissues generally depend on the quality of tissue submitted for RT-PCR (or other mRNA based assays as described earlier). It remains the most critical aspect of this technology, requiring special tissue/cell handling to prevent its degradation especially by endogenous RNases. Tissues have to be snap-frozen within minutes after surgery and never allowed to warm up to inhibit endogenous RNases. The presence of mRNA for a cytokine in tissue is meaningful but its absence is not, as cytokine transcripts are short-lived, and their absence in the tested tissue could reflect rapid processing or degradation rather than the lack of synthesis.

3.5. Cytokine micro-arrays

3.5.1. DNA microarrays

The widening use of DNA chips or microarrays allows for simultaneous analysis of expression of thousands of genes from various tissues or cells at mRNA level (99). This technology can be used to determine the up regulation of genes expressing cytokines in response to external signals, cellular stress and various pathologic conditions (100). The microarray is a micro-hybridization based assay. In analogy to hybridization assays, it involves a microfilter or a chip made of a porous membrane or materials such as glass, plastic, silicon, gold, or gel, in which either oligonucleotides or cDNA fragments are spotted or synthesized at high density (for example, 10,000 per cm^2) (101,102). Probes for the microarray can be complementary DNA (cDNA), RNA, genomic DNA, or plasmid libraries, which are appropriately labeled and hybridized to the chip (103). To measure the resulting hybridization signals, radioactive and fluorescence detection strategies are used. The result is an image obtained by fluorescence scanner or phosphorimager and that can be processed with computer software to generate a spreadsheet of gene expression values (Figure 4). In today's era, where cytokine profiling is gaining much more importance, cytokine microarrays can be used to understand changes in cytokine milieu of specific cell types or tissues in response to external signals. Indeed global gene expression profiles have been used to demonstrate the involvement of key cytokines in inflammatory and immune responses (104-106). Although there has been an increase in the availability of commercially available microarrays, the technology is still restricted to research applications.

3.5.2. Protein Microarrays

Similar to gene arrays, the protein microarrays are useful in expression profiling of proteins including

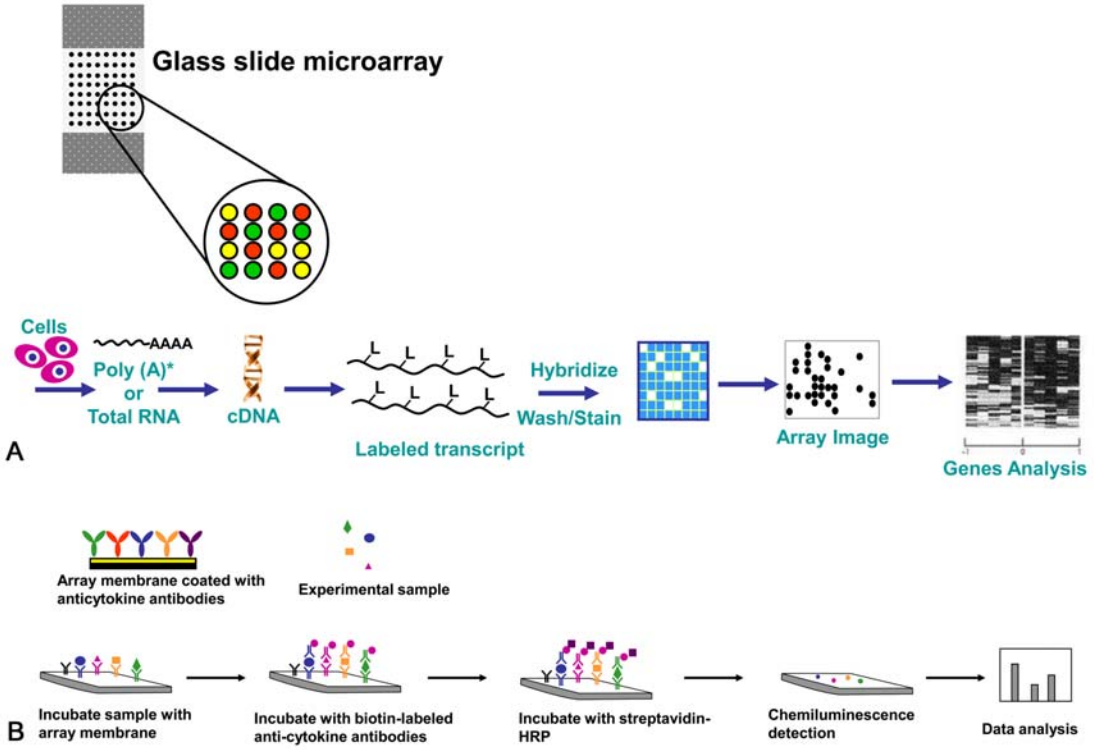

Figure 4. Detection of cytokines expressed in biological specimens by DNA microarrays (4a) and Protein microarrays (4b). Both the technologies have made detection of several cytokines at a time from small quantities of biological specimens quite feasible.

cytokines, their receptors, inhibitors or proteins involved in the cascade of cytokine synthesis from cells or tissues. Even though detection of expressed proteins is always preferred by biologists as diagnostic markers and targets of therapeutic intervention, protein expression profiling is technically more difficult than nucleic acid based technologies, since protein samples are often very limited in supply and unlike nucleic acids, cannot be amplified (107). Moreover, antibody microarrays have much more limitations in multiplexing than DNA microarrays, since cross reactivity seem to impinge, no matter what analytes are involved. Mass spectrometry, which relies on identification of proteins on the basis of their unique mass/charge (m/z) ratios, offers one method for comparing proteins isolated from biological samples obtained from individuals with different pathological conditions (108). However the disadvantage of this technology is its low throughput and difficulty in molecular identification of markers, besides high costs.

With the ability of miniaturization of multiplex immunoassays for measuring biological analytes, protein microarrays have gained popularity more recently (109-111). Schweitzer *et al* (112) demonstrated a microarray system with antibodies that can be used for simultaneous measurement of 75 analytes. Commercial development of protein microarray in clinical diagnostics has really picked up during the past couple of years, making it one of the most exploratory fields in clinical diagnostics. Commercially antibody arrays with hundreds of distinct antibodies are now available from BD Biosciences Clontech (Palo Alto, CA) and Hypromatrix (Millbury, MA). Antibody based cytokine microarrays can be successfully employed to analyze many cytokines simultaneously with enhanced sensitivities of chemiluminescence and high specificities of conventional enzyme immunoassays using minimum amount of samples (113). At our laboratory too we have successfully assessed a biochip array system (Evidence Investigator, Randox Laboratotories Ltd, Crumlin, UK) to measure 12 cytokines in Immunological and virological discordant HIV-1 infected subjects to understand differences in their cytokine profiles as compared to concordant HIV-1 infected individuals. The discordant patients in our pilot study showed significantly higher levels of VEGF and EGF as compared to concordant patients ($p<0.05$), besides showing elevated levels of IL-1β. The results of biochip array were confirmed by ELISA that showed similar comparison between the two groups of patients in terms of plasma VEGF and EGF (114). A similar cytokine array system is available from R&D Systems that is capable of measuring 36 different cytokines, chemokines and acute phase proteins in a single sample using a chemiluminiscence based detection (115). More recently, Ray Biotech Inc. announced the release of a glass slide based antibody array that is capable of detecting up to 174 cytokines, chemokines and growth factors in a single experiment using as little as 10 µl of sample (116). Such reports clearly indicate that expression profiling of proteins by microarray has tremendous applications in the future where clinicians and scientists can generate vast amount of information from the patient samples not only in identification of disease

markers but also develop their understanding of the disease process. Amidst their growing scope, reproducibility of multiplex assays needs consideration since many of the recent assays have shown poor precision and higher coefficients of variation (117). Multiplexing of many analytes at the same time also influences the overall sensitivity of individual analytes. Moreover, due to involvement of large numbers of analytes in miniaturized formats, the assays become prone to cross contamination and give false positive results. Thus, the multiplex technologies require extensive validation especially in clinical settings before they make their mark in routine practice.

4. OTHER UPCOMING TECHNOLOGIES

Besides microarray, there have been recent reports in the literature on other new technologies for quantitation of cytokines. One of such techniques is the gel micro-drop (GMD) secretion assay that is particularly useful for measurement of cytokines from a single cell (118). It involves encapsulating single cells in a biotinylated agarose matrix, addition of a streptavidin bridge, diffusion of a biotinylated capture antibody followed by detection of secreted molecules using a fluorescently labeled reporter antibody. Drukier *et al* (119) have described an ultra sensitive immunoassay using multi-photon detection that could be highly useful in diagnostic proteomics of blood. The technique combines immunoassays with multi-photon-detection read-out of ^{125}I, enhancing the sensitivity in femtograms/ml. The technique has been validated for quantitation of TNF-α, VEGF, IL-1β, IL-6 and IL-8 from human serum. More recently, a hybrid technique that involves flow cytometric analysis of cytokines using multi-analyte fluorescence microarray technology has been described (120). The technique is quite similar to cytometric bead array and can detect upto 10 cytokines from a single sample. Another assay for cytokines using aptamers has also been described recently. Aptamers are short nucleic acid sequences that are used as ligands to bind their targets with high affinity. Specifically, for the determination of cytokines and growth factors, several assays making use of aptamers have been developed, including aptamer-based ELISA, fluorescence (anisotropy and resonance energy transfer) assays and proximity ligation assays (121).

5. PERSPECTIVE

Presently, the cytokine assays are still restricted to the research laboratories and have not become widely acceptable in routine diagnostic investigations. This can be attributed to the fact that cytokine biology is quite complex and many cytokine assays, especially the upcoming technologies require significant efforts in standardization, performance characterization as well as development of proficiency testing programs for quality assurance (122). Amidst the availability of various techniques to measure cytokines in biological specimens, rationale for selecting the right assay becomes critical. Overall, there are two strategies by which cytokines can be assessed in biological specimens; first strategy is direct measurement, using one of the various assays either in a monoplex or multiplex format, while the second strategy involves measurement of cytokine activities (Cytokine bioassays). Simple techniques like ELISA or ELISPOT are still widely popular and are most used by the clinical research laboratories. Another important factor that should be considered is the source of specimen and its processing, since complications in the processing of specimens too add to the variability in the data generated. For example, mRNA based methods or inducible cytokine production assays following cell separation procedures like magnetic separation are technically difficult to perform and can influence the assessment of cytokines. Since the cytokine milieu is quite different in blood, tissues or other body compartments, it is ultimately the user, who is best to decide the type of specimen required, depending on the type of information needed. It is a well established fact that, combined effect of multiple cytokines and chemokines in the immune response to disease is often more important than the function of one specific cytokine. Therefore, measurement of multiple cytokines in biological fluids provides more relevant information on their milieu. With the advent of microarray technology, miniaturization of immunoassays and reduction in the cost of reagents cytokine profiling is becoming more feasible now. It seems likely that expression profiling of cytokines will have tremendous applications in the understanding of various disease processes, pathological conditions and their utility as bio-therapeutic agents.

6. REFERENCES

1. G. R. Guy, N. S. Bee & C. S. Peng: Lymphokine signal transduction. *Cell Signal* 2, 415-425 (1990)
2. J. Kang & S. D. Der: Cytokine functions in the formative stages of a lymphocyte's life. *Curr Opin Immunol* 16, 180-190 (2004)
3. J. A. Bienvenu, G. Monneret, M. C. Gutowski & Fabien N: Cytokine assays in human sera and tissues. *Toxicology* 129, 55-61 (1998)
4. S. Romagnani: T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* 85, 9-18 (2000)
5. F. Brombacher, R. A. Kastelein & A. G. Novel: IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 24, 207-212 (2003)
6. C. A. Hunter: New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 5, 521-531 (2005)
7. J. C. Renauld: New insights into the role of cytokines in asthma. *J Clin Pathol* 54, 577-89 (2001)
8. M. Stassen, S. Fondel, T. Bopp, T. C. Richter, C. Muller & J. Kubach *et al*: Human CD25+ regulatory T cells: two subsets defined by the integrins alpha 4 beta 7 or alpha 4 beta 1 confer distinct suppressive properties upon CD4+ T helper cells. *Eur J Immunol* 34, 1303-1311 (2004)
9. H. B. Oral, S. V. Kotenko, M. Yilmaz, O. Mani, J. Zumkehr, K. Blaser *et al*: Regulation of T cells and cytokines by the interleukin-10 (IL-10)-family cytokines IL-19, IL-20, IL-22, IL-24 and IL-26. *Eur J Immunol* 36, 380-388 (2006)
10. R. Ruckert, K. Brandt, A. Braun, H. G. Hoymann, U. Herz, V. Budagian *et al*: Blocking IL-15 prevents the

induction of allergen-specific T cells and allergic inflammation *in vivo*. *J Immunol* 174, 5507-5515 (2005)
11. M. Kawaguchi, M. Adachi, N. Oda, F. Kokubu & S. K. Huang: IL-17 cytokine family. *J Allergy Clin Immunol* 114, 1265-1273 (2004)
12. W. R. Coward, A. Marei, A. Yang, M. M. Vasa-Nicotera & S. C. Chow: Statin-induced proinflammatory response in mitogen-activated peripheral blood mononuclear cells through the activation of caspase-1 and IL-18 secretion in monocytes. *J Immunol* 176, 5284-5292 (2006)
13. T. Hideshima, K. Podar, D. Chauhan & K. C. Anderson: Cytokines and signal transduction. *Best Pract Res Clin Haematol* 18, 509-524 (2005)
14. D. Asthana & M. A. Fletcher: Cytokines and chemokines in clinical immunology. *Clin Immunol Newslett* 17, 61-62 (1997)
15. K. Goodkin & D. Asthana: The influence of cytokines and chemokines on the pathophysiology of HIV-1 associated cognitive motor disorders. *Clin Immunol Newslett* 17, 62-65 (1997)
16. T. L. Whiteside: Cytokine assays. *Biotechniques* 33, S4-S15 (2002)
17. J. R. Crowther: ELISA. Theory and Practice. *Methods Mol Bio* 42, 1-223
(1995)
18. C. A. Dinarello: ELISA kits based on monoclonal antibodies do not measure total IL-2 synthesis. *J Immunol Methods* 148, 255-259 (1992)
19. M. Svenson, M. B. Hansen, P. Heegaard, K. Abell & K. Bendtzen. Specific binding of interleukin 1 (IL-1) beta and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine* 5, 427-435 (1993)
20. P. Galle, M. Svenson, K. Bendtzen & M. B. Hansen: High levels of neutralizing IL-6 autoantibodies in 0.1% of apparently healthy blood donors. *Eur J Immunol* 34, 3267-3275 (2004)
21. L. L. Carter & S. L. Swain: Single cell analyses of cytokine production. *Curr Opin Immunol* 9, 177-182 (1997)
22. D. Wallace, A. Hildesheim & L. A. Pinto. Comparison of bench top micro plate beta counters with the traditional gamma counting method for measurement of chromium-51 release in cytotoxic assays. *Clin Diagn Lab Immunol* 11, 255–260 (2004)
23. J. Slack, J. E. Sims, A. M. Pitt & S. K. Dower: Application of the multiscreen system to cytokine radioreceptor assays. *Biotechniques* 7, 1132-1138 (1989)
24. L. J. Kricka: Chemiluminescent and bioluminescent techniques. *Clin Chem* 37, 1472-1481 (1991)
25. A. Meager: Measurement of cytokines by bioassays: theory and application. *Methods* 38, 237-252 (2006)
26. A. Meager, The Molecular Biology of Cytokines. *Wiley*, Chichester, UK (1998)
27. A. Meager, Cytokines: interleukins. Encyclopedia of Molecular Cell Biology and Molecular Medicine, *Wiley-VCH*, Weinheim, Germany 3, 115–151 (2004)
28. A. Meager, Viral inhibitors and immune response mediators: the interferons. Encyclopedia of Molecular Cell Biology and Molecular Medicine, *Wiley-VCH,* Weinheim, Germany 15, 387–421 (2005)
29. H. Ibelgaufts: Cytokines Online Pathfinder Encyclopedia (COPE). www.copewithcytokines.de/.
30. R. Thorpe, M. Wadhwa, C. Page & A. Mire-Sluis. Bioassays for the characterization and control of therapeutic cytokines; determination of potency. *Dev Biol Stand* 97, 61–71 (1999)
31. American Type Culture Collection (ATCC). www.atcc.org.
32. A. Meager: Seventh WHO informal consultation on standards for cytokines, growth factors and endocrinological substances. *Biologicals* 32,105–111 (2004)
33. WHO Expert Committee on Biological Standardization (ECBS). www.who.int/biologicals.
34. D. G. Remick, Protein analysis and bioassays of cytokines and cytokine receptors. Manual of Clinical Laboratory Immunology, *ASM Press* Washington, DC 6, 320-337 (2002)
35. S. Gillis, M. M. Ferm, W. E. Ou & K. A. Smith: T cell growth factor: parameters of production and a quantitative microassay for activity. *J Immunol* 120, 2027 (1978)
36. M. Higuchi, S. Singh & B. B. Aggarwal: Characterization of the apoptotic effects of human tumor necrosis factor: development of highly rapid and specific bioassay for human tumor necrosis factor and lymphotoxin using human target cells. *J Immunol Methods* 178,173-181 (1995)
37. T. Mosmann: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63 (1983)
38. H. Tada, O. Shiho, K. Kuroshima, M. Koyama & K. Tsukamoto: An improved colorimetric assay for interleukin 2. *J Immunol Methods* 93,157-165 (1986)
39. L. A. Randall, M. Wadhwa, R. Thurpe & A. R. Mire-Sluis: A novel sensitive bioassay for transforming growth factor-b. *J Immunol Methods* 164, 61-67 (1993)
40. A. R. Mire-Sluis & R. Thorpe. Laboratory protocols for the quantitation of cytokines by bioassay using cytokine responsive cell lines. *J Immunol Methods* 211,199-210 (1998)
41. R. E. Callard, J. G. Sheilds & S. H. Smith, Assays for human B cell growth and differentiation factors - Lymphokines and Interferons. A Practical Approach. *IRL Press*, Oxford, 345-364 (1987)
42. M. D. Sadick, M. X. Sliwkowski, A. Nuijens, L. Bald, N. Chaing, J. A. Lofgren *et al*: Analysis of heregulin-induced ErbB2 phosphorylation with a high throughput kinase receptor activation enzymelinked immunosorbant assay. *Anal Biochem* 235, 207-214 (1996)
43. C. R. Yu, K. W. Peden, M. B. Zaitseva, H. Golding & J. M. Farber: CCR9A and CCR9B: two receptors for the chemokine CCL25/TECK/Ck beta-15 that differ in their sensitivities to ligand. *J Immunol* 164, 293-305 (2000)
44. U. E. Gibson & S. M. Kramer: Enzyme-linked bio-immunoassay for IFN-g by HLA-DR induction. *J Immunol Methods* 125,105-131 (1989)
45. J. M. Schroder, U. Mroweitz, E. Morita & E. Christophers: Purification and partial biochemical characterisation of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. *J Immunol Methods* 139, 3474-3483 (1987)

46. A. Doyle, M. Stein, S. Keshav & S. Gordon, Assays for macrophage activation by cytokines. Cytokines: A Practical Approach. *IRL Press* Oxford, 269-278 (1995)
47. W. G. Junger, T. A. Cardoza, F. C. Liu, D. B. Hoyt & R. Goodwin: Improved rapid photometric assay for quantitative measurement of PMN migration. *J Immunol Methods* 160, 73 (1993)
48. D. D. Taub, M. L. Key, D. Clark & S. M. Turcovski-Corrales: Chemotaxis of T lymphocytes on extracellular matrix proteins. Analysis of the *in vitro* method to quantitate chemotaxis of human T cells. *J Immunol Methods* 184,187-198 (1995)
49. D. D. Taub, P. Proost, W. J. Murphy, M. Anver, D. L. Longo, Van-Damme J *et al*: Monocyte hemotactic protein-1 (MCP-1)-2 and -3 are hemotactic for human T lymphocytes. *J Clin Invest* 95, 1370-1376 (1995)
50. K. Konishi, F. Tanabe, M. Taniguchi, H. Yamauchi, T. Tanimoto, M. Ikeda *et al*: A simple and sensitive bioassay for the detection of human interleukin-18/interferon-gamma-inducing factor using human myelomonocytic KG-1 cells. *J Immunol Methods* 209,187-191 (1997)
51. C. A. Dinarello, D. Novick, A. J. Puren, G. Fantuzzi, L. Shapiro, H. Muhl *et al*: Overview of interleukin-18: more than an interferon-gamma inducing factor. *J Leukocyte Biol* 63, 658-664 (1998)
52. J. A. Lewis, Antiviral activity of cytokines - Cytokines. A Practical Approach. *IRL Press* Oxford, 129-141 (1995)
53. J. A. Lewis: A sensitive biological assay for interferons. J Immunol Methods 185, 9-17 (1995)
54. E. Pungor, J. G. Files, J. D. Gabe, L. T. Do, W. P. Foley, J. L. Gray *et al*: A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. *J Interferon Cytokine Res* 18, 1025–1030 (1998).
55. P. Gallay, J. P. Mach & S. Carrel: Characterization and detection of naturally occurring antibodies against IL-1 alpha and IL-1 beta in normal human plasma. *Eur Cytokine Network* 2, 329-338 (1991)
56. L. T. May, H. Viguet, J. S. Kenney, N. Ida, A. C. Allison & P. B. Sehgal: High levels of "complexed" interleukin 6 in human blood. *J Biol Chem* 267, 19698-19704 (1992)
57. M. J. Bargetzi, M. Lantz, C. G. Smith, F. M. Torti, I. Olsson, S. P. Eisenberg *et al*: Interleukin 1-b induces interleukin 1 receptor antagonist and tumor necrosis factor binding protein in humans. *Cancer Res* 53, 4010-4013 (1993)
58. D. A. A. Vignalli: Multiplexed particle-based flow cytometric assays. *J of Immunol Methods* 243, 243-255 (2000)
59. H. R. Hill & T. B. Martin: The flow cytometric analysis of cytokines using multi-analyte fluorescence microarray technology. *Methods* 38, 312-316 (2006)
60. M. J. Loza, J. S. Faust & B. Perussia: Multiple color immunofluorescence for cytokine detection at the single-cell level. *Mol Biotechnol* 23, 245-258 (2003)
61. L. D. Giavedoni: Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology. *J of Immunol Methods* 301, 89-101 (2005)
62. C. Heijmans-Antonissen, F. Wesseldijk , R. J. Munnikes , F. J. Huygen, P. van der Meijden, W. C. Hop *et al*: Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. *Mediators Inflamm* 1, 28398 (2006)
63. H. T. Maecker : Human CD81 directly enhances Th1 and Th2 cell activation, but preferentially induces proliferation of Th2 cells upon long-term stimulation. *BMC Immunol* 4, 1 (2003)
64. K. Uchimura, M. Itoh, K. Yamamoto, S. Imamura, M. Makino, T. Kato *et al* : The effects of CD40- and interleukin (IL-4)-activated CD23+ cells on the production of IL-10 by mononuclear cells in Graves' disease: the role of CD8+ cells. *Clin Exp Immunol* 128, 308-312 (2002)
65. A. McNeill, J. Kastrup & B. T. Backstrom: A simplified cytokine immunoassay using magnetic polymer particles. *Scand J Immunol* 60, 287-291 (2004)
66. G. Gilliand, S. Perrin, K. Blanchard & H. F. Bunn. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 87, 2725-2729 (1990)
67. S. R. Carding D. Lu & K. Bottomly: A polymerase chain reaction assay for the detection and quantification of cytokine gene expression in small numbers of cells. *J Immunol Methods* 151, 277-287 (1992)
68. N. Kruse, M. Pette, K. Toyka & P. Rieckman: Quanitfication of cytokine mRNA expression by RT-PCR in samples of previously frozen blood. *J Immunol Methods* 210,195-203 (1997)
69. U. E. Gibson, C. A. Heid & P. M. Williams: A novel method for real time quantitative RT-PCR. *Genome Res* 6, 995-1001 (1996)
70. C. A. Heid, J. Stevens, K. J. Livak & P. M. Williams: Real time quantitiative PCR. *Genome Res* 6, 986-994 (1996)
71. P. Boeuf, I. Vigan-Womas, D. Jublot, S. Loizon, J. Barale, B. Dicky *et al*: CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. *BMC Immunol* 6, 5 (2005)
72. C. A. Gogos, E. Drosou, H. P. Bassaris & A. Skoutelis: Pro-verus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 181,176-180 (2000)
73. M. Vukmanovic-Stejic, B. Vyas, P. Gorak-Stolinska, A. Noble & D. M. Kemeny: Human Tc1 and Tc2/Tc0 CD8 t-cell clones display distinct cell surface and functional phenotypes. *Blood* 95, 231-240 (2000)
74. A. L. Dent, A. L. Shaffer, X. Yu, D. Allman & L. M. Staudt: Control of inflammation, cytokine expression and germinal center formation by Bcl-6. *Science* 276, 589-592 (1997)
75. R. Sallusto, D. Lenig, C. R. Mackay & A. Lanzavecchia: Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 187, 875-883 (1998)
76. C. E. Lewis & A. Campbell, Visualizing the production of cytokines and their receptors by single human cells - Cytokines: A Practical Approach. *IRL Press*, Oxford, 339-356 (1995)
77. T. S. Hawley & R. G. Hawley: Flow Cytometry Protocols, 2nd ed, Vol 263. New Jersey: Humana Press, 2004.
78. A. Letsch & C. Scheibenbogen: Quantification and characterization of specific T-cells by antigen-specific

cytokine production using ELISPOT assay or intracellular cytokine staining. *Methods* 31,143-149 (2003)
79. S. K. Arora: Analysis of intracellular cytokines using flowcytometry.
Methods Cell Sci 24, 37-40 (2002)
80. L. J. Picker, M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser *et al*: Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86, 1408-1419 (1995)
81. A. D. Sarr, L. Salako & S. Mboup: Prospects for an HIV Vaccine. http://www.apin.harvard.edu/Chapter20.pdf
82. C. Czerkinsky, G. Andersson, H. P. Ekre, L. A. Nilsson, L. Klareskog & O. Ouchterlony: Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of g-interferon-secreting cells. *J Immunol Methods* 110, 29-36 (1988)
83. W. Herr, J. Schneider, L. W. Ansgar, E. Wandel, K. H. Meyer zum Buschenfelde & T. Wolfel: Detection and quantification of blood derived CD8+ T lymphocytes secreting tumor necrosis factor-a in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J Immunol Methods* 191,131-142 (1996)
84. W. Herr, B. Linn, N. Leister, E. Wandel, K. H. Meyer zum Buschenfelde & T. Wolfel: The use of computer-assisted video image analysis for the quantitation of CD8+ T lymphocytes producing tumor necrosis factor-a spots in response to peptide antigens. *J Immunol Methods* 203,141-151 (1997)
85. T. Asai, W. J. Storkus & T. L. Whiteside: Evaluation of the modified ELISPOT assay for interferon-g production in cancer patients receiving anti-tumor vaccines. *Clin Diagn Lab Immunol* 7,145-154 (2000)
86. J. Bennouna, A. Hildesheim, K. Chikamatsu, W. Gooding, W. J. Storkus & T. L. Whiteside: Application of IL-5 ELSIPOT asssays to quantification of antigen-specific T helper responses. *J Immunol Methods* 261,145-156 (2002)
87. N. Meidenbauer, D. T. Harris, L. E. Spitler & T. L. Whiteside: Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. *Prostate* 43, 88-100 (2000)
88. N. Meidenbauer, W. Gooding, L. E. Spitler, D. Harris & T. L. Whiteside: Recovery of z chain expression and changes in spontaneous IL-10 produciton after PSA-based vaccines in patients with prostate cancer. *Br J Cancer* 86,168-178 (2002)
89. Y. Okamoto & M. Nishida: Dual-color ELISPOT assay for analyzing cytokine balance. *Methods Mol Biol* 302, 263-272 (2005)
90. Y. Okamoto, T. Abe, T. Niwa, S. Mizuhashi & M. Nishida: Development of a dual color enzyme-linked immunospot assay for simultaneous detection of murine T helper type 1- and type 2- cells. *Immunopharmacology* 39,107-116 (1998)
91. A. Y. Karulin, M. D. Hesse, M. Tary-Lehmann & P. V. Lehmann: Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. *J Immunol* 164, 1862-1872 (2000)
92. S. Palzer, T. Bailey, C. Hartnett, A. Grant, M. Tsang & A. E. Kalyuzhny : Simultaneous detection of multiple cytokines in ELISPOT assays. *Methods Mol Biol* 302, 273-288 (2005)
93. A. Gazagne, W. Malkusch, B. Vingert, W. H. Fridman & Tartour E: Fluorospot assay: methodological analysis. *Methods Mol Biol* 302:289-296 (2005)
94. B. Sander, J. Andersson & U. Andersson: Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol Rev* 119, 65-93 (1991)
95. G. L. Chupp, E. A. Wright, D. Wu, M. Vallen-Mashikian, W. W. Cruikshank, D. M. Center *et al*: Tissue and T cell distribution of precursor and mature IL-16. *J Immunol* 161, 3114-3119 (1998)
96. R. Thorpe, M. Wadhwa, C. Bird & A. R. Mire-Sluis: Detection and measurement of cytokines. *Blood Rev* 6, 133-148 (1992)
97. D. Vitolo, T. Zerbe, A. Kanbour, C. Dahl, R. B. Herberman & T. L. Whiteside: Expression of mRNA for cytokines in tumor-infiltrating mononuclear cells in ovarian adenocarcinoma and invasive breast cancer. *Int J Cancer* 51, 573-580 (1992)
98. D. Vitolo, A. Kanbour, J. T. Johnson, R. B. Herberman & T. L. Whiteside: *In situ* hybridization for cytokine gene transcripts in the solid tumor microenvironment. *Eur J Cancer* 3, 371-377 (1994)
99. J. R. Pollack, C. M. Perou, A. A. Alizadeh, M. B. Eisen, A. Pergamenschikov, C. F. Williams *et al*: Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* 23, 41- 46 (1999)
100. C. Napoli, F. de Nigris & V. Sica: New advances in microarrays: finding the genes causally involved in disease. *Methods Mol Med* 108, 215-233 (2005)
101. M. Schena, D. Shalson, R. W. Davis & P. O. Brown: Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470 (1995)
102. M. Chese, R. Yang, E. Hubbell, A. Berno, X. C. Huang, D. Stern *et al*: Accessing genetic information with high-density DNA array. *Science* 224, 610–614 (1996)
103. D. E. Gray, S. C. Case-Green, T. S. Fell, P. J. Dobson & E. M. Southern: Ellipsometric and interferometric characterization of DNA probe immobilized on a combinatorial array. *Langmuir* 13, 2833–2842 (1997)
104. Z. Wang, D. Neuburg, C. Li, L. Su, J. Y. Kim, J. C. Chen *et al*: Global gene expression profiling in whole-blood samples from individuals exposed to metal fumes. *Environ Health Perspect* 113, 233-241 (2005)
105. M. Gatzka, R. Piekorz, R. Moriggl, J. Rawlings, J. .N. Ihle: A role for STAT5A/B in protection of peripheral T-lymphocytes from postactivation apoptosis: insights from gene expression profiling. *Cytokine* 34, 143-154 (2006)
106. H. Crandall, D. M. Dunn, Y. Ma, R. M. Wooten, J. F. Zachary, J. H. Weis *et al*: Gene expression profiling reveals unique pathways associated with differential severity of lyme arthritis. *J Immunol* 177, 7930-7942 (2006)
107. D. S. Wilson & S. Nock: Recent developments in protein microarray technology. *Angew Chem Int Ed Engl* 42, 494-500 (2003)
108. E. F. Petricoin, A. M. Ardekani, B. A. Hitt, P. J. Levine, V. A. Fusaro, S. M. Steinberg *et al*: Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359, 572-577 (2002)

109. P. J. Utz: Protein arrays for studying blood cells and their secreted products. *Immunol Rev* 204, 264-282 (2005)
110. B. A. McKinney, D. M. Reif, M. T. Rock, K. M. Edwards, S. F. Kingsmore & J. H. Moore *et al*: Cytokine Expression Patterns Associated with Systemic Adverse Events following Smallpox Immunization. *J Infect Dis* 194, 444-453 (2006)
111. M. J. Zilliox, Parmigiani G & D. E. Griffin: Gene expression patterns in dendritic cells infected with measles virus compared with other pathogens. *Proc Natl Acad Sci U S A* 103, 3363-3368 (2006)
112. B. Schweitzer, S. Roberts, B. Grimwade, W. Shao, M. Wang , Q. Fu *et al*: Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nat Biotechnol* 20, 359-365 (2002)
113. S. P. Fitzgerald, J. V. Lamont, R. I. McConnel & E. O. Benchikh: Development of a high-throughput automated analyzer using biochip array technology. *Clin Chem* 51,1165-1176 (2005)
114. N. Sachdeva, H. S. Yoon, K. Oshima, L. Cintron, D. Garcia, K. Goodkin *et al*: Analysis of plasma cytokines in Immunological and virological discordant HIV-1 infected patients by microarray system: A pilot study." In: 14th International symposium on HIV and Emerging Infectious Diseases, Toulon, France, 21-23 June 2006. www.isheid.com.
115. New Products. Proteome Profiler™ Arrays human cytokine array. R&D Systems New Products. June 2006.
116. Ray Biotech News. http://www.raybiotech.com/news.asp. December 2005.
117. W. L. Roberts: AHA Conference Proceedings: CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease. *Circulation* 110, e572-576 (2004).
118. O. Atochina, R. Mylvaganam, Y. Akselband & P. McGrath: Comparison of results using the gel microdrop cytokine secretion assay with ELISPOT and intracellular cytokine staining assay. *Cytokine* 27, 120-128 (2004)
119. A. K. Drukier, N. Ossetrova, E. Schors, L. R. Brown, J. Tomaszewski, R. Sainsbury & J. Godovac-Zimmermann: Ultra-sensitive immunoassays using multi-photon-detection in diagnostic proteomics of blood. *J Proteome Res* 4, 2375-2378 (2005)
120. H. R. Hill & T. B. Martins: The flow cytometric analysis of cytokines using multi-analyte fluorescence microarray technology. *Methods* 38, 312-316 (2006)
121. J. W. Guthrie, C. L. Hamula, H. Zhang & XC Le: Assays for cytokines using aptamers. *Methods* 38, 324-330 (2006)
122. J. L. Fahey, N. Aziz, J. Spitzler, S. Plaeger, P. Nishanian, J. L. Lathey, J. Seigel, A. L Landay, R. Kilarui, J. L. Schmitz, C. White, D. W. Wara, R. Akridge, J. Cutili, S. D. Douglas, J. Reuben, W. Shearer, M. Nokta, R. Pollard, R. Schooley, D. Asthana, Y Mizrachi and M. Waxdal: Need for an external proficiency testing program for cytokines, chemokines and plasma markers of immune activation. Clin. Diag. Lab. Immunol. 7 (4), 540-548 (2000)

Abbreviations: ATCC, American type culture collection; BSF-3, B-cell stimulating factor 3; cDNA, complementary DNA; CFSE, carboxy fluoroscein succinimidyl ester; CyProQuant-PCR, Cytokine profiling quantitative PCR; ECBS, expert committee on biological standardization; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbant assay; ELISPOT, enzyme-linked immunospot assay; FGF, fibroblast growth factor; GM-CSF, granulocyte macrophage-colony stimulating factor; HRP, horseradish peroxidase; ICC, Intra-cytoplasmic cytokine staining; IFN-γ, interferon gamma; IGF-1, insulin-like growth factor 1, IL, interleukin; IRMA, immunoradiometric assay; ISH, *In situ* hybridization; MCP-1, monocyte chemotactic protein-1; MHC, major histocompatibility complex; MIP-1α, macrophage-inflammatory protein-1 alpha; MTT tetrazolium, 3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide; NIH, national institute of health; NK cells, natural killer cells; OD, optical density; RANTES, regulated on activation normal T cell expressed and secreted ; RIA, Radioimmunoassay; RPA, RNase protection assays; RRA, radioreceptor assays; rRNA, ribosomal RNA; RT-PCR, reverse transcriptase linked – polymerase chain reaction; SDF-1α, stromal cell-derived factor 1alpha; TGF-β, transforming growth factor beta; TMB, tetra methyl benzidine; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; WHO, world health organization.

Key Words: Cytokines, quantitation, technologies, ELISA, Chemiluminescence, ELISPOT, RT-PCR, Multi parameteric flow cytometry, Immunostaining, Microarray.

Send correspondence to: Dr Deshratn Asthana, Laboratory for Clinical and Biological Studies, University of Miami-Miller School of Medicine, 1550 NW 10th Ave, Fox Building, Suite -118 Miami, FL 33136, Tel: 305-243-2010, Fax: 305-243-2013, E-mail: desh@miami.edu

http://www.bioscience.org/current/vol12.htm