In this issue: FLOW CYTOMETRY IN THE CLINICAL LABORATORY

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Measurement of soluble biomarkers by flow cytometry

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Abstract

Microparticle based flow cytometric assays for determination of the level of soluble biomarkers are widely used in several research applications and in some diagnostic setups. The major advantages of these multiplex systems are that they can measure a large number of analytes (up to 500) at the same time reducing assay time, costs and sample volume. Most of these assays are based on antigen-antibody interactions and work as traditional immunoassays, but nucleic acid alterations – by using special hybridization probes –, enzyme- substrate or receptor-ligand interactions can be also studied with them. The applied beads are nowadays provided by the manufacturers, but cheaper biological microbeads can be prepared by any user. One part of the systems can be used on any research or clinical cytometers, but some companies provide dedicated analyzers for their multiplex bead arrays. Due to the high standardization of the bead production and the preparation of the assay components the analytical properties of these assays are quite reliable with a wide range of available applications. Cytokines, intracellular fusion proteins, activated/phosphorylated components of different signaling pathways, transcription factors and nuclear receptors can be identified and quantitated. The assays may serve the diagnostics of autoimmune disorders, different viral and bacterial infections, as well as genetic alterations such as single nucleotide polymorphisms, small deletions/insertions or even nucleotide triplet expansions can be also identified. The most important principles, technical details and applications of these systems are discussed in this short review.

Introduction

Changes in the concentration of different proteins in human serum or plasma may indicate the presence of several normal or pathological processes and show the progression of different disorders. The measurement of total serum protein and its subfractions or quantitation of individual proteins have been applied in routine laboratory diagnostics for several decades. The first assays determined the total protein content of serum using mainly protein-specific dyes and spectrophotometry, while the subfractions were analyzed by electrophoresis. Later, serum proteins were studied based on their enzymatic activity as these molecules were easily measured by the conversion of their substrate to a colored product measured by photometry. A major step was the introduction of antibody-antigen based immunoassays that could considerably enhance the number of tested individual proteins. Several different types of immunoassays were then developed for measuring the light scatter alteration caused by the immunocomplexes formed due to the antibody-antigen interactions (turbidimetry, nephelometry). Furthermore, a variety of methods was introduced that used antigens or antibodies labeled with radioactive, enzyme, fluorescence or luminescence components in competitive or sandwich immunoassays. Several of these tests were applied on automated immunoanalyzers enhancing the efficacy and the precision of these assays. In spite of all advantages the major drawback of these methods is the measurement of only one single analyte at one time that increases the time period and sample volume

required for analysis. Multiplex immunoassays can solve these problems. One possibility is the development of antibody-based protein chips – like the RANDOX QuantiPlasma 69 system – where antibodies are coated to small carriers (chips) and can measure large number of proteins – 69 plasma proteins in that case – simultaneously in a "sandwhich" or competitive way. The other option is the application of multicolor flow cytometric bead arrays.

The introduction of microparticles into flow cytometry opened a brand new field for determination of the level of soluble biomarkers in different human body fluids. The method is very robust, and the standardized production of the microbeads provides a very reproducible and accurate technique. The introduction of multicolor beads further enhanced its applicability, since each type (color) of microparticles supports an individual test, and thus a large number of assays can be run at the same time. Besides high-throughput, the flexibility of the systems is also excellent as different vendors offer high freedom for the customers in choosing the proper bead mixes for the proper clinical/research solutions. Another advantage of the technique is the versatility of the system, since several biomolecules and markers can be tested on the same platform from proteins to nucleic acids and from enzyme-substrate interactions to receptor-ligand binding [1-4].

Basic principles of the microparticle based flow cytometric assays

Protein determination - immunoassays

The most widely used application of the system is based on antigen-antibody interaction and works as most of the classical immunoassays. The solid base is provided by the fluorescently labeled microparticles, and in the "two-site" or "sandwich" type of this assay a capture antibody is coated on them. This antibody recognizes the serum protein of interest and the detection of the captured protein is managed by a fluorescently labeled second antibody (Figure 1A). The second type of the immunoassays based on the competition of different assay components (competitive immunoassays). One option is when a capture antibody is coated on the beads and a fluorescently labeled antigen is competing with the appropriate "cold" antigens of the tested sample. The higher the amount of the protein in the serum the lower the signal we can detect. In case of another type the tested antigen is immobilized on the surface of the beads, and their soluble analogues in the tested sample compete for binding to a fluorescently labeled antigen-specific antibody (Figure 1B). The microparticles make also possible the detection of autoantibodies when autoantigens are present on the surface of the beads. The bound autoantibodies are identified by a secondary anti-human immunoglobulin specific antibody labeled by a second/third fluorescent dye. Similarly, microbe-specific antibodies can be identified supporting the rapid diagnostics of different bacterial/viral/fungal infections (Figure 1C) [1-4].

Nucleic acid detection – hybridization

Another possible application of the system is the detection of certain nucleic acid sequences based on the hybridization to oligonucleotide probes coated on the microparticles. Such an assay makes possible for instance the identification of single nucleotide polymorphisms (SNPs) or point mutations in the tested sample. The target region of the genomic DNA is amplified in a specific PCR reaction using fluorescently labeled primers and then the single stranded PCR products are hybridized to the probes present on the beads. In this case two types of beads capture the labeled DNA; one carries an oligonucleotide containing the wild type nucleotide of the SNP, while the other bead carries the mutant one. The positivity/negativity and the fluorescent intensity of the two beads measured will define the proper genotype. The system can also support gene-expression studies. The RNA extracted from e.g. two differently treated cell populations can be transcribed into cDNA, and one of the cDNA samples can be simultaneously labeled fluorescently. The beads of the multiplex system contain special probes for special genes, and the competition between the two cDNA samples for binding to these probes will provide information about the relative expression of these genes in the two differently treated samples (Figure 1D) [4-5].

Enzyme-substrate and receptor-ligand assays

A more research and development orientated application of the system is the search for proper substrates or ligands for certain enzymes or receptors. These assays can work simply using fluorescently labeled test ligands and receptor coated particles, or a competitive assay is also applicable, when known ligand(s) of the receptor labeled fluorescently compete with the new test molecules for binding to receptors coated onto the microparticles (Figure 1E, F) [6,7].

Types of the microparticles

The most widely used microparticles are plastic beads that can be easily manufactured with a high throughput and a large precision. The latest technologies also allow the color-coding up to 500 different bead entities; furthermore, the size of the beads can be also a usable variable in creating more-and-more complex arrays. Certain companies also incorporate the possibility for magnetic separation of the beads in their systems [1-3].

Concerning the utility of these beads one part of the assays produced by different companies are predefined, because well-defined capture antibodies are coated on the surface of the microparticles. Thus, these beads are suitable for measuring only one type of an analyte. Another option is the purchase of "multifunctional" beads coated by free carboxyl or amino groups

suitable for covalent adhesion of certain proteins according to the design of the user. Avidin/streptavidin or goat anti-mouse immunoglobulin label can also support the labeling of the beads by user defined molecules and antibodies [2].

Historically, other easily accessible bioparticles were also applied in similar microparticle assays. More than a decade ago a simple flow cytometric test was developed by our group that could measure the serum soluble CD14 (sCD14) concentration in an easy way. The membrane bound CD14 of isolated human monocytes competed with sCD14 in the serum of the tested patients for the binding to a fluorescently labeled anti-CD14 monoclonal antibody. The fluorescence intensity of the monocytes was measured by flow cytometry and the lower the fluorescence we observed the higher the sCD14 concentration was in the serum. A serial dilution of a serum sample with known concentration of sCD14 served as calibrator for the assay (Figures 1B and 2) [8].

The application of normal human cells as target microparticle itself might also supply the detection of different autoantibodies (such as anti-neutrophil cytoplasmic antibodies). Moreover, certain microbes can help the identification of specific antimicrobial antibodies thought to be important in the diagnostics of special immune-mediated disorders (e.g. in colitis ulcerosa or Crohn's disease). A typical example is the evaluation anti-Saccharomyces cerevisiae antibodies (ASCA) using bakers' yeast suspension as a substrate particle of the detection. In these cases the gates are set around the target bioparticles based on their scatter properties and the fluorescence of the secondary anti-human immunoglobulin antibody is measured (Figure 1C) [2].

A rather novel approach is to create low cost and easily reproducible bioparticles (mainly bacteria and fungi) coated by avidin/streptavidin for capture of biotin-labeled antibodies, antigens or even special nucleotide probes. The fluorescent labeling of these microbes is easy handled and even multiplex labeling is achievable that makes this type of particles a real alternative especially in research applications [4].

Single versus Multiplex systems

The prototype of these systems was developed using only single beads that did not differ much from single ELISA-s in terms of their throughput and efficacy. A large breakthrough was the introduction of multiple beads with different sizes and then with different labeling color. In the recent systems the microparticles have a certain color that identifies the assay (and the protein detected by the beads) and in the case of multiplex systems each type of bead (recognizing different soluble markers) has a

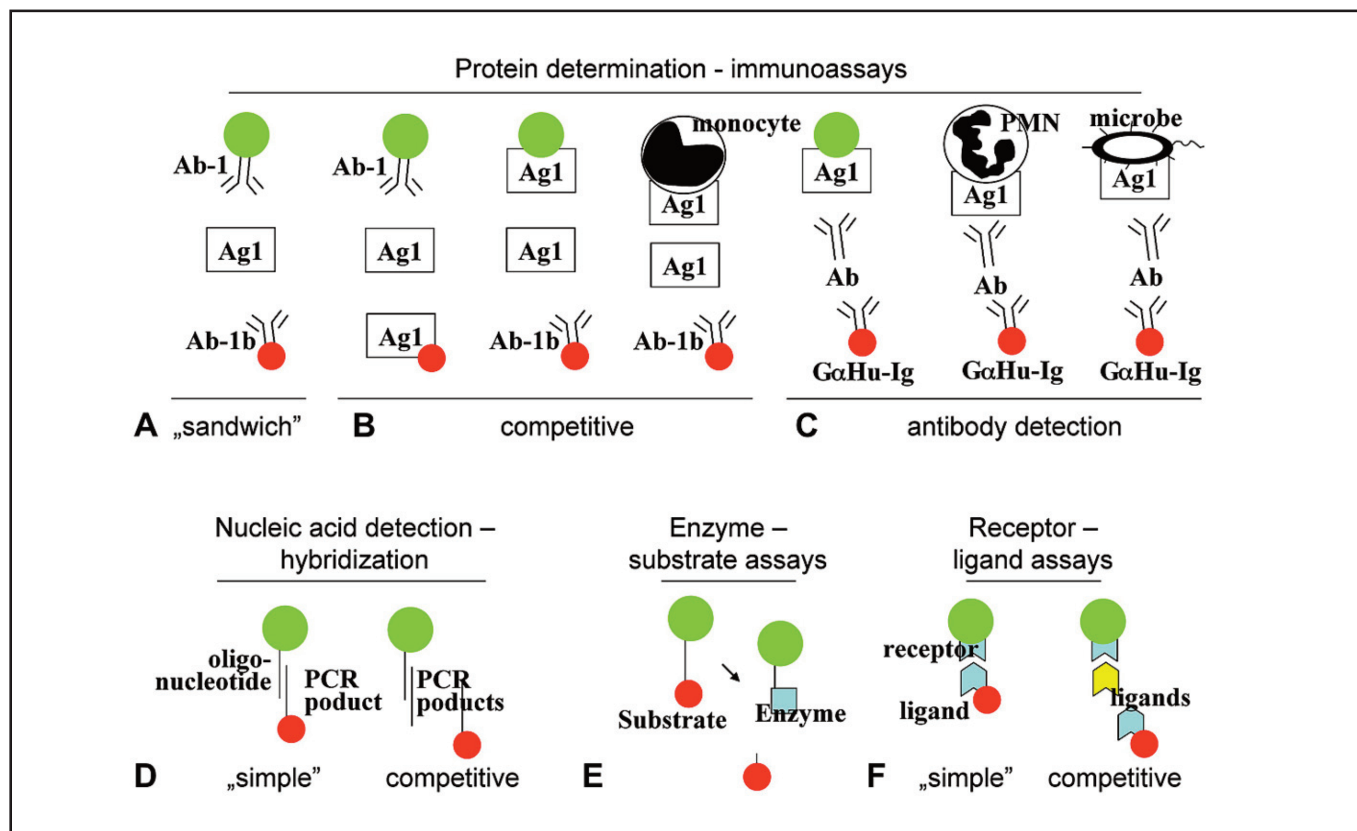

Figure 1
Basic principles of the microparticle based flow cytometric assays. Green circles: fluorescently labeled microparticles; red circles: fluorophore used for detection; Ag: antigen; Ab: antibody; GaHu-Ig: Goat anti-human immunoglobulin specific antibody.

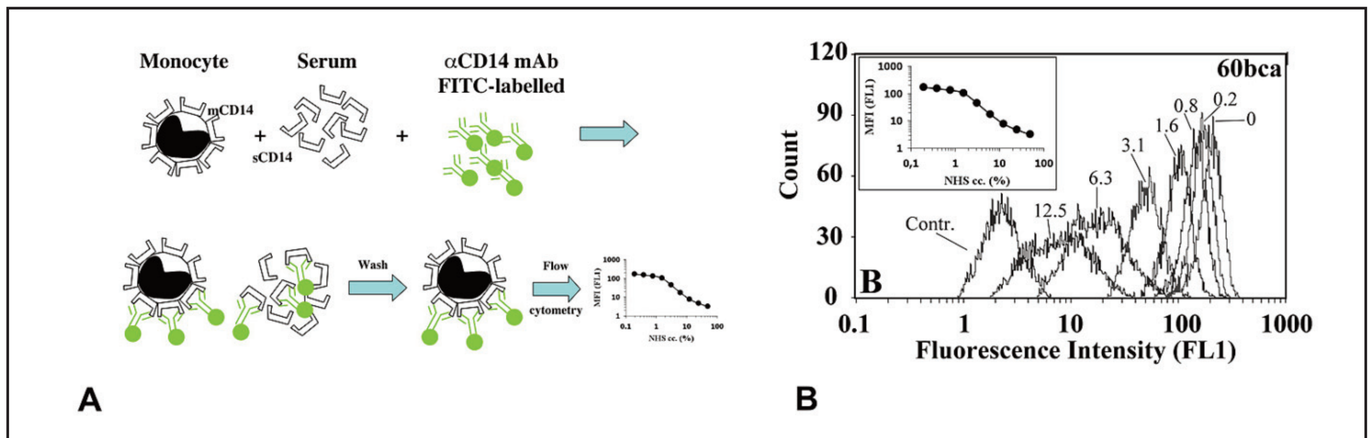

Figure 2

Determination of sCD14 in human serum by a simple flow cytometry based competitive immunoassay. Isolated monocytes were incubated with the appropriate dilution of the tested serum and a FITC-labeled anti-CD14 monoclonal antibody. The cells were washed and the fluorescence intensity of the monocytes – gated based on their scatter properties – was analyzed by a Coulter EPICS XL flow cytometer (A). As the sCD14 in the serum competes with the mCD14 of the monocytes for binding to the labeled anti-CD14 antibody, the higher the concentration of sCD14 in the sample the lower the fluorescence intensity we detected on monocytes. A serial 2-fold dilution of a serum sample containing known amount of sCD14 served as standard. The representative FL1 histograms of the standard samples and the standard curve created from these data is presented on Figure 2B.

separate color. The detection antibody is labeled by another fluorophore that helps the detection and quantitation of the captured protein. In multiplex systems the color of the beads – measured in one or two fluorescence channels of the flow cytometer – identifies the measured protein, while the fluorescence intensity, provided by the dye of the detection antibody on a separate fluorescence channel, clarifies the amount of protein bound to a certain bead. In each assay of the multiplex system where we gate on a single bead population the fluorophore of the detection antibody can be the same in the case of all detected proteins. The amount of protein in the tested sample can be accurately quantitated using standard samples containing known amounts of the tested protein. Obviously, in case of multiplex systems one standard curve is required for each measured analyte [1-3].

One possible limitation of the multiplex system is the different dynamic range of tested analytes. In the case of a certain experimental setup such as in the stimulation of isolated cells the amount of one analyte released from cells can be very high, that require the dilution of the sample, while the others have to be measured in the undiluted supernatant. As nowadays the flexibility of the assays is very high – with proper pilot experiments the over- or under- expressed analytes can be identified and – the matching tests can be freely selected. Another important issue is the optimization of the assay conditions (washing buffer, pH, ionic strength) that is suitable for each antigen-antibody pair. Moreover, the multiplexing of the assays always results in the elevation of the background noise and the decrease in the sensitivity of the tests that has to be kept in mind. Finally, the possible interactions of certain antibodies or the so called "matrix effect" can alter the properties of individual tests. Because of these effects, an assay that is running properly on its own will not be automatically reliable in a multiplex system [1,2].

Detection platforms

One part of the multiplex flow cytometric bead assays can be used with everyday clinical or research flow cytometers. The Becton-Dickinson BD™ Cytometric Bead Array (CBA) system supports the majority of Coulter, DAKO, Partec and Becton-Dickinson flow cytometers. The kit includes the appropriate data for the setup of the equipment, reagents, calibrators and even an analysis software that can evaluate the list mode data of the measurement in an Excel-base format. Another approach is offered by the Luminex's xMAP Technology. In that case the company developed special flow cytometers designed for multiplex bead analysis. The Luminex 100/200™ and FLEXMAP 3D® systems have similar components like any flow cytometers but they can be used only for measurement and evaluation of the xMAP multiplex bead arrays. A very similar approach is offered by Bio-Rad's Bio-Plex MAGPIX, Bio-Plex 200, and Bio-Plex 3D systems. In contrast to these instruments that identify the beads based on their fluorescent labels the Copalis (Coupled Particle Light Scattering) system of Diasorin discriminates between single beads with different diameters and aggregated beads based on their scatter properties [1-3].

Possible applications

The number of possible tests available in the form of multiplex bead assay is radically increasing. Several companies provide

Possible diagnostic use

Protein detection
- Acut phase proteins
- Autoantibodies
- Bone metabolism markers
- Cancer markers
- Cardiac markers
- Cytokines and chemokines
- Growth factors
- Hormones
- Immunoglobulins and isotypes
- Inflammation
- Metabolic markers
- Microbiology (antigen, antibody detection)

Nucleic acid detection
- Mutation identification
- Microbiology (nucleic acid detection)

Research use

Protein detection
- Adhesion
- Angiogenesis
- Apoptosis
- Signal transduction
- Transcription factors
- Nuclear receptors
- Neurobiology
- Kidney toxicity
- Enzyme-substrate interactions
- Receptor-ligand binding

Nucleic acid detection
- Gene expression analysis
- SNP identification
- MicroRNA evaluation

Figure 3
Possible applications of the flow cytometric multiplex bead assays.

a long range of tests covering different areas of research and diagnostic use. These possible applications are listed in Figure 3. One of the first areas that is still the most widely used application of the cytometric bead array systems is the measurement of different cytokines in body fluid of patients and controls or in the supernatant of differently stimulated cells. The "cytometric bead array cytokines" search in the PubMed database provides 300 hits and the first publication was prepared in 2001, describing the simultaneous measurement of 6 cytokines in tear samples [9]. Nowadays assays are available for IL-1 to IL-18, TNFα and β, INFα, β and γ, more than 10 chemokines and soluble cytokine receptors. In addition, complex kits are available for Th1/Th2/Th17 (IL-2, IL-4, IL-6, IL-10, IFNγ, TNFα, IL-17α) or inflammatory (IL-6, IL-10, IL-12, IFNγ, TNFα, MCP-1) cytokines [1-3]. Figure 4 illustrates one of our experiments aiming the multiplex determination of cytokines in LPS stimulated whole blood and platelet rich plasma (PRP).

The detection of intracellular proteins in cell lysates is also a valuable technique that can serve diagnostic and research purposes, too. The recently developed assay for measuring the bcr/abl fusion protein in the lysate of white blood cells is a unique method for the rapid diagnosis of chronic myeloid or acut lymphoblastic leukaemia. The assay is based on fluorescent beads coated with monoclonal antibodies that can recognize the bcr part of the bcr/abl fusion proteins independently of the type of the fusion (the minor or the major breakpoint cluster region of the bcr gene is involved in the translocation). The lysate of the white blood cells is mixed with the beads and then a fluorescently-labeled anti-abl antibody detects the bound proteins [10]. In a recent work, we evaluated the analytical properties of this assay. The intra- assay CV% of positive controls was respectable as 3.7% was in the normal and 10% was found in the pathological range. The cut-off for mean fluorescence intensity was 112 that provided 100% sensitivity and 100% specificity for the assay. The results of the cytometric bead assay showed 100% agreement with the molecular biological tests used for bcr/abl transcript detection [11]. Recently, a similar assay was introduced for the detection of the PML/RARA fusion protein [12].

Another broad field within the detection of intracellular proteins is the identification of activated/phosphorylated components of different signaling pathways. Dozens of signaling molecules can be tested including the MAPK-family, Wnt/GSK/Akt or the JAK/STAT pathway in addition to the activation of several growth factor receptors (like EGFR, IGFR, VEGFR, c-kit, c-Met) [1,3]. Several publications are also available in the international literature. Koeper and colleagues described a skin implant model to test the toxic/irritating effect of different skin sensitizers by simultaneous testing the phosphorylation of the MAP- kinases, STAT1

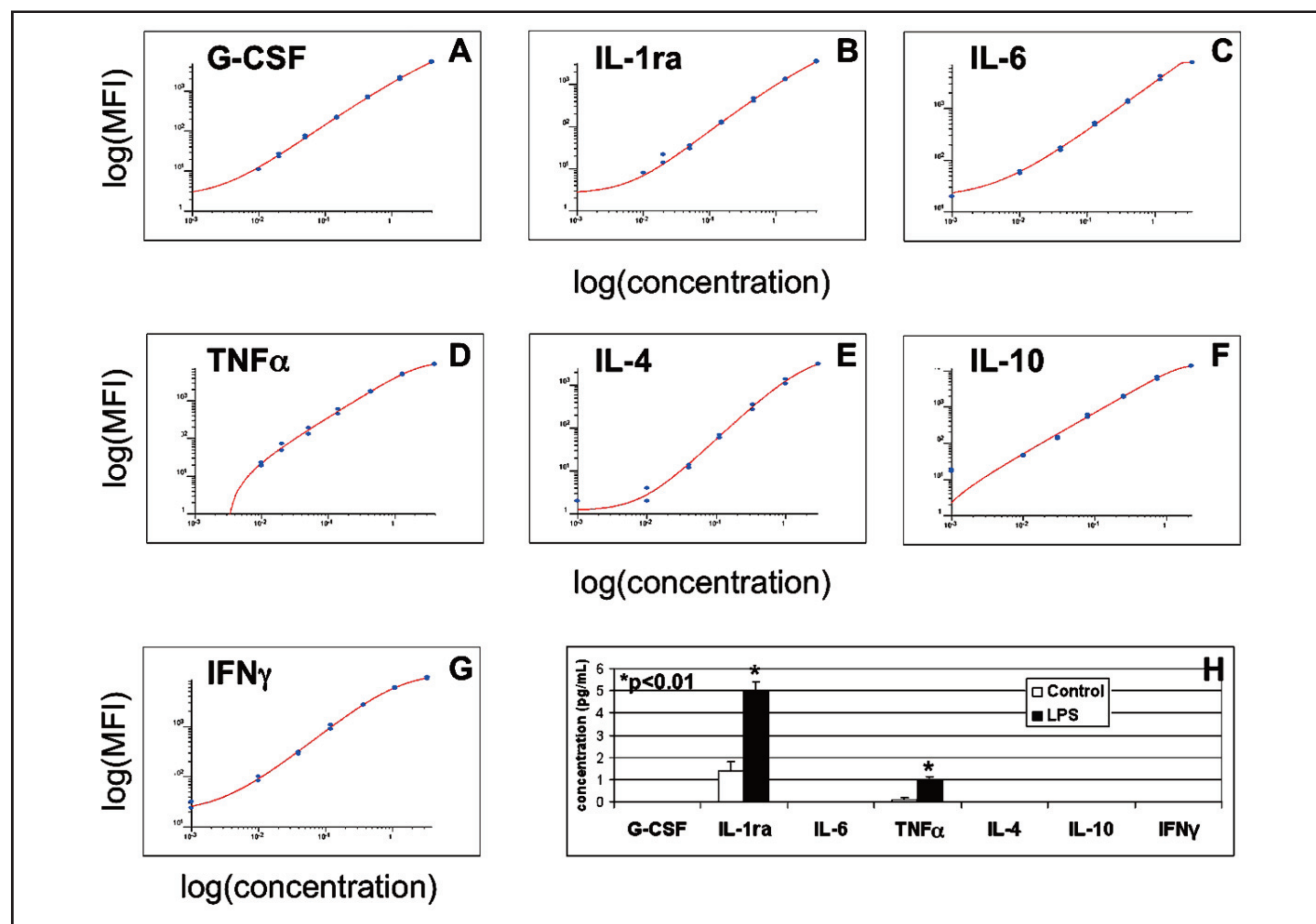

Figure 4
Multiplex detection of human cytokines in LPS-stimulated whole blood and PRP.
Citrated whole blood or PRP samples were stimulated by Re-LPS (10 μg mL^{-1}) for 1 hour at 37°C and G-CSF, IL-1ra, IL-6, TNFα, IL-4, IL-10 and IFNγ levels were determined simultaneously. Briefly, microparticles with pre-coated antigen-specific antibodies on their surface were added to the samples and pipetted into wells on a microplate. For the analysis of the levels of microparticle-bound antigen, a biotinylated secondary antibody and a streptavidin-PE conjugate were applied. After microparticles were suspended in buffer, results were determined by a Luminex 100™ analyzer (Luminex, Austin, TX, USA). One laser was microparticle-specific to show which antigen level was under investigation, and another laser determined the fluorescent signal, which was directly proportional to the concentration of antigen bound. On Figure 3A-G we can see the standard curves of each cytokine measured. Figure 3H presents the concentration of each cytokines in the plasma of LPS stimulated whole blood. There was a significant increase (P<0.01) in the level of two cytokines due to the Re-LPS stimulation versus the control sample (IL-1ra: 4.98±0.42 pg mL^{-1} vs. 1.42±0.39 pg mL^{-1}) (TNF-α: 1.0±0.12 pg mL^{-1} vs. 0.1±0.06 pg mL^{-1}). The cytokine concentrations in the PRP were below the detection levels independently of LPS stimulation.

and Phospholipase C γ [13]. Dawes et al. studied the activation of the pERK, pP38, and pJNK upon TGFβ2 stimulation in lens epithelial cells during the differentiation to myofibroblasts [14], while Wong and colleagues investigated the MAPK and NF-κB activity in IL-25 stimulated T helper lymphocytes [15]. Similarly, intracellular nuclear receptors can be also detected using the multiplex bead arrays like in the work of Schneiderhan-Marra, where 56 proteins – including oestrogen receptor – were tested in breast cancer needle biopsy samples [16].

A very dynamically developing field is the use of the multiplex bead arrays for identification of valuable biomarkers in the early diagnostics or follow-up of malignant disorders [1]. Opstal-van Winden et al. tested simultaneously 10 biomarkers in the serum of breast cancer patients but they could not identify a panel that could help the early diagnosis of this disorder [17]. Kim and coworkers analyzed 3 markers (CA-125, transthyretin, and apolipoprotein A1) of ovarian cancer in a multiplex system and could show that the combination of these markers was superior to the analytical performance of the individual ones [18]. In a very recent elegant study, 30 biomarkers were tested in the serum of non-small cell lung cancer patients using a multiplex bead array. Twenty-three parameters differed between the controls and the patients, and the combined application of the 5 highest-ranked biomarkers (α1-antitrypsin, CYFRA 21-1, IGF-1, RANTES, AFP) could discriminate between controls and NSCLC patients with high accuracy [19].

For the diagnostics of autoimmune disorders a relatively large number of multiplex assays are already available. The complex evaluation of antinuclear antibodies (ANA), extractable nuclear antigens (ENA) or anti-neutrophil cytoplasmic antibodies (ANCA) can be performed using these systems. Furthermore, disease specific panels are also available for celiac disease, systemic lupus erythematosus, autoimmune thyroid disorders and vasculitis (1,20,21). An import application of the technique is the HLA-typing of donors and recipients of kidney transplantation and the identification of donor-specific serum antibodies [1,22].
The application of multiplex bead assays can support diagnostic microbiology, too [1]. Yu et al. developed an inhibitory multiplex bead assay to determine 26 serotypes of pathogenic *Streptococcus pneumoniae* strains [23], while Wagner and colleagues described an assay that can simultaneously identify antibodies specific for the outer surface protein A, C and F of *Borrelia burgdorferi* serving the diagnostics of Lyme disease in both humans and animals [24]. Furthermore, food poisoning caused by *Escherichia coli* O157:H7 can be identified easily and rapidly using this multiplex bead system [25]. Commercially available arrays are ready to use for Epstein-Barr and Herpes virus identification, and for respiratory tract viruses and measles/mumps/rubeola/varicella detection [1].
A promising new are of this field is the combination of multiplex bead arrays with the DNA/RNA based molecular biologic techniques. The system is suitable to analyze the presence of SNPs like the IL-6 SNP distribution in different ethnic groups [26] or the 22 SNPs of the ABC transporter genes in healthy individuals [27]. It can also detect small insertions or deletions in the BRCA1 gene [5] or even the number of nucleotide triplet expansions in Huntington disease [5]. Furthermore, the cytometric array can be used for gene expression studies [28].

Discussion

The development of microbeads based flow cytometric assays for measuring soluble biomarkers started more than 10 years ago. At that time we thought that this system would make a revolution in laboratory diagnostics as covering even several hundreds of analytes in one run could replace the currently available laboratory techniques. The system is indeed a robust one, the number of the available assays is enormous and there are certain areas – especially in research – where this technique really became a number one choice. On the other hand, the still high costs of the systems and also the evolution of laboratory automation and the development of the classical laboratory tests did not let the change occur. Nevertheless, these microparticle based assays are very much useful, and will support our research and certain diagnostic activities in the future.

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