

# 25

## Bead-Based Flow Cytometric Assays: A Multiplex Assay Platform with Applications in Diagnostic Microbiology

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### Introduction

Researchers have focused on developing specific assays for conclusively identifying and measuring the levels of bacteria, fungi, protozoa, viruses (microbes), and their associated products (biomarkers) that cause disease in humans and animals (Murray et al., 2003). Traditional methods using microscopy and chemical or immunological stains, test cultures with selective media or target cells, or serological assays have been used effectively to identify infectious agents in biological specimens or environmental samples. However, due to increasing veterinary, medical, and public health concerns, faster and more accurate diagnostic tools have been sought. Multiplex array-based assays allow for a range of biomarkers to be rapidly and simultaneously measured within specimens (Robertson and Nicholson, 2005). Recently, multiplex bead-based flow cytometric immunoassays have been developed and applied that show great promise for improving the study, diagnosis, and therapeutic management of infectious diseases (Alvarez-Barrientos et al., 2000; Jani et al., 2002).

The advent of genetic and molecular engineering and monoclonal antibody generation has driven the development of many assays that employ immunoassay-based technology for specifically measuring various biomarkers. Highly purified recombinant or synthetic molecules have been used as antigens to generate specific antibody preparations and to provide target antigens or calibrated standards for these assays. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) are widely used for specific and sensitive analyte measurements that have diagnostic value (Andreotti et al., 2003). Although this methodology is well suited for single-analyte analysis in a relatively large sample volume, methods that simultaneously quantitate multiple analytes from a relatively small sample size are needed to improve the speed, utility, and confidence of diagnostic assays. The combination of traditional immunoassay-based technologies with multiparameter flow cytometry has promoted the development of bead-based, high-throughput multiplex immunoassays. In this method, polymer microspheres (beads) that are

several micrometers in diameter replace the traditional immunoassay plates and serve as the solid phase for the flow cytometric assay (Horan and Wheeless, 1977). Many different types of bead-based flow cytometric immunoassays have been described including those that have indirect or sandwich immunoassay formats (McHugh et al., 1994; Vignali, 2000).

The indirect immunoassay format is especially useful for measuring specific antibody levels in biological fluids (e.g., for determining rising or falling serum antibody titers during the course of an infectious disease). In this format, capture beads are coated or covalently conjugated with target antigen molecules (Fig. 25.1A). For diagnostic microbiology assays, these may be either crude microbial extracts or highly purified molecular preparations. The beads are mixed with test biological fluids (tissue culture supernatants, serum, wound exudates, bronchial lavage fluids) that may contain specific antibodies. Solutions or samples containing quantified levels of antibodies that are specific for the analyte of interest can serve as the assay's calibration standards. Adding fluorescent anti-immunoglobulin detection antibodies (DAb) develops the assay. These fluorescent DAb may be specific for all immunoglobulin isotypes. In these assays, total antibody levels are measured. Alternatively, the DAb may be specific for immunoglobulin isotypes and thus allow measurement of antibodies with IgM, IgA, IgG, or IgE isotypes. Isotype-specific measurements of antibody levels may be very important for monitoring the course of an infectious disease and for characterizing the nature of immune responses to vaccinations (Wild, 2001). The levels and isotype profile for antibodies may indicate whether the humoral immune response is weakly or strongly protective or nonprotective for a particular infectious disease.

In the sandwich immunoassay format, specific capture antibodies can be noncovalently adsorbed or covalently bound to plastic beads (Fig. 25.1B). The capture beads are then mixed with biological fluids that contain target analytes of interest (e.g., soluble microbial antigens including structural molecules, enzymes, or toxins). During or after incubation of the capture beads with the samples, a fluorescent DAb (or biotinylated DAb developed with a fluorescent avidin conjugate) is added that serves as the reporter fluorophore. The analyte levels in bead-based flow cytometric immunoassays are proportional to the mean fluorescence intensity (MFI) levels generated by the bead/analyte-bound fluorescent DAb. With the inclusion of a standard antigen of known concentration, such as natural, synthesized, or recombinant molecules, standard dilution curves can be generated from which the corresponding levels of target analyte are interpolated.

Bead-based single analyte flow immunoassays were first described for measuring the levels of  $\alpha$ -fetoprotein (Frenge et al., 1993) and beta2-microglobulin (Bishop and Davis, 1997). Multiplex bead-based flow cytometric immunoassays were made possible by using beads of different sizes. McHugh (1994) and Fulwyler et al. (1988) used beads of different sizes as carriers for target antigens or antibodies. The beads were differentiated by their sizes that were related to their forward light-scattering characteristics determined by flow cytometric analysis. Appropriate electronic gating strategies were used during data acquisition and

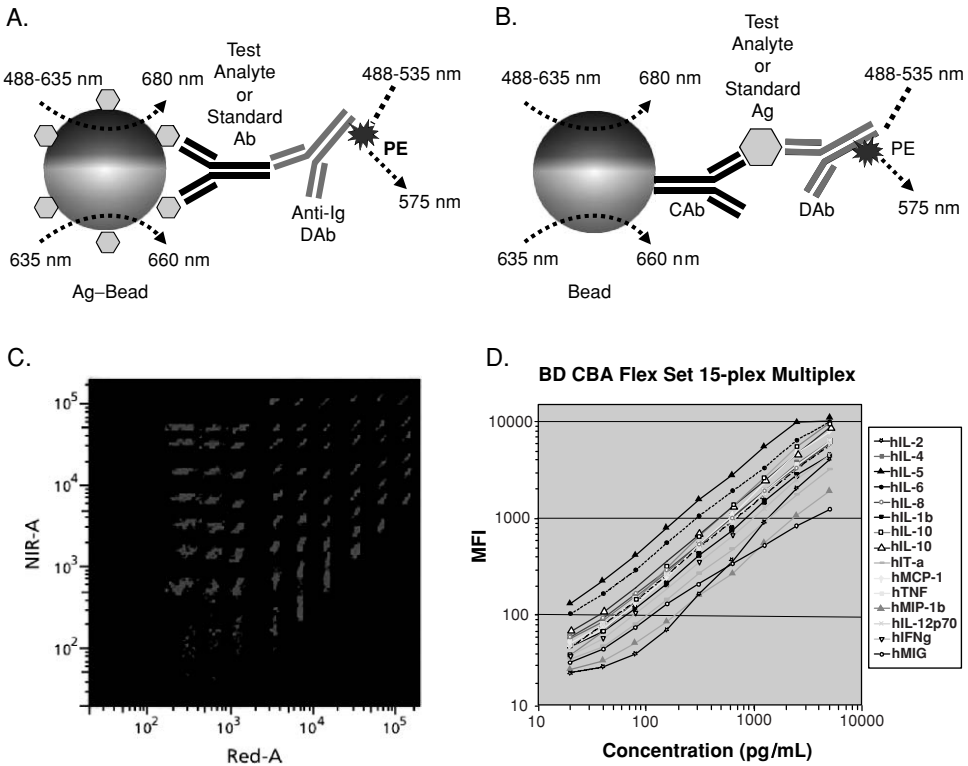


FIGURE 25.1. Multiplex flow cytometric immunoassays. Multiplex flow cytometric immunoassay systems using a BD Cytometric Bead Array (CBA) Flex Set format are shown. Beads dyed with various levels of two different fluorescent dyes are stylistically represented in the top panels. Panel A: For indirect flow cytometric immunoassay format, beads are coated or conjugated with target antigens. When mixed with specimens or calibrated solutions, antibodies specific for the target antigen bind to the capture beads and in turn are recognized by fluorescent anti-immunoglobulin reagents. Upon flow cytometric analysis, two incident light beams of different wavelengths excite the two fluorescent bead dyes. The beads emit fluorescent signals that are distinguished in terms of their wavelength (depending on the type of dye) and fluorescence intensity (depending on the incorporated dye level). Panel B: Alternatively, in flow cytometric sandwich immunoassays, the dyed beads are conjugated with a specific capture antibody (CAb). When mixed with specimens or standard solutions, the capture beads specifically bind and localize antigens to their surface. Fluorescent detection antibodies (DAb) are added that bind to another antigen site. Panel C: A large number of different bead positions can be resolved in two-color dot plots, for example, the logarithmically amplified FL3 and FL4 channels of a BD FACSCalibur flow cytometer or the NIR and Red channels of a BD FACSArray bioanalyzer (72 different bead sets). Beads with different two-color fluorescent positions can be combined in assays to create a multiplex assay. Incident light causes the fluorescent DAb that is directly conjugated with a fluorochrome such as phycoerythrin (PE) to emit distinct fluorescence signals. These reporter signal intensities are commensurate with the amount of analyte-bound DAb connected with distinct fluorescent capture bead sets. The flow cytometric data can then be reanalyzed to generate standard curves and to quantify the levels of specific analytes in test specimens. Panel D: Standard curves generated from a 15-plex Cytokine CBA Flex Set analysis are shown.

reanalysis to determine the mean fluorescent intensities emitted by the fluorescent DAb associated with each distinct capture bead group.

Multiplex flow cytometric immunoassays can also be made using beads of the same size with unique fluorescence properties. In these assays, distinct capture bead sets are produced by labeling them with different levels of one type of fluorescent dye. The different capture bead sets can then be distinguished by flow cytometric analysis based on their distinct MFI. Fluorescent DAb provide the second type of fluorescence that constitutes the reporter immunoassay signals (Camilla et al., 1998). This approach is useful for creating low-complexity bead-sets (Chen et al., 1999). However, larger distinguishable bead sets can be prepared by labeling them with distinct proportions of two fluorescent dyes (Fulwyler et al., 1988), for example, red and far-red dyes as detected by a flow cytometer (Fig. 25.1C). In this case, DAb coupled to a third fluorescent dye [e.g., phycoerythrin (PE)] generate the immunoassay signals. With this approach, it is possible to develop a number of different capture bead/DAb pairs. These pairs are initially developed and tested separately for their sensitivity and range of analyte measurement. As part of the validation process, the pairs are mixed together with other bead pairs and tested for their capacities to specifically measure analytes in mixtures such as serum samples or cocktails of diluted standards. Capture bead/DAb pairs must be capable of specifically measuring the same analyte levels when tested separately or in mixtures. With the selection of good antibody pairs that do not cross-react with other analytes or antibodies in the system, flow cytometric immunoassays can be used to measure the levels of multiple analytes (e.g., biological markers associated with one or more microorganisms).

The bead-captured analyte levels measured by multiplex flow cytometric immunoassays are proportional to the mean fluorescence intensity levels generated by the bead/analyte-bound fluorescent DAb. With the inclusion of calibrated analyte solutions (e.g., solutions that contain target antigens or antibodies of known concentration), standard dilution curves can be generated from which the corresponding levels of target analytes are interpolated. Data reanalysis using immunoassay software provides a selection of a variety of standard curve-fitting choices. These allow for the best fit of immunoassay signals as a function of standard and sample concentrations. These may include either linear-log, log-log, or four- or five-parameter transformations (Fig. 25.1D).

Multiplex flow cytometric immunoassays are ideal for measuring biomarkers associated with multiple infectious agents (Jani et al., 2002). This may be particularly important in complex diseases such as the generalized acquired immunodeficiency caused by the human immunodeficiency virus (HIV). Immunodeficient individuals are susceptible to infections caused by multiple opportunistic pathogens. Alternatively, multiple biomarkers associated with a particular infectious agent can be analyzed for a conclusive diagnosis or prognosis. In the latter case, biomarkers for incubation, acute, convalescent, and recovery phases from a disease caused by an infectious agent can be carefully monitored.

Multiplex flow cytometric immunoassays simplify analyte profile determinations. Only a single sample is needed to detect and quantify a number of analytes,

an advantage when the sample volume is limited (e.g., pediatric or cerebrospinal fluid samples). The multiple independent measurements within each bead population assure good precision. The high sensitivity and wide detection range afforded by logarithmically amplified fluorescent signals or high-resolution digital data systems is another advantage (e.g., fewer sample dilutions are required). Moreover, the greater surface area and exposure to soluble analytes improves the bead-based assay kinetics when compared with traditional microwell plate-based immunoassays.

In the following sections, some examples of multiplex bead-based flow cytometric immunoassays as applied to diagnostic microbiology are presented. These include methods for measuring the levels of specific antibodies generated by hosts to specific microbial pathogens as well as detection of the microbial pathogens and their effector molecules. In addition, the potential use of these multiplex assays for measuring biological response modifiers such as cytokines and chemokines that are produced as a consequence of infection, leading to disease states in some instances, are presented.

## Flow Cytometry-Based Assays for Detection of Microbe-Specific Antibodies and Microbial Antigens

### *Measurement of Antimicrobial Antibody Responses*

Flow cytometric immunoassays are well suited to monitor antibody responses against antigens from infectious agents. Determinations of stable, rising, or dropping antibody titers and the immunoglobulin isotype of the antimicrobial antibodies provide valuable information concerning the nature or status of an infection or the efficacy of an antimicrobial therapy or vaccine. A growing list of publications for this technology is being reported (Jani et al., 2002).

Best and colleagues (1992) successfully developed an indirect bead assay for the serodiagnosis of *Helicobacter pylori*. For this assay, the investigators coated plastic microspheres with a crude multicomponent antigen mixture prepared from *H. pylori* to react with antibodies that were present in patient sera. Fluorescein-conjugated anti-human IgG DAB were used to measure the levels of IgG anti-*H. pylori* antibodies that were present in serum samples from infected (positive by culture or histological examination) or noninfected control patients. Pooled negative sera served to determine the assay's limit of detection and relative serum antibody titers with weakly and strongly positive control sera included in each assay to control for intra- and inter-assay variability. The bead-based assay was 100% sensitive and 89% specific (positive and negative predictive values of 90% and 100%) and gave no equivocal results. Parallel testing with a commercial ELISA was 96% sensitive and 89% specific (positive and negative predictive values of 90% and 96%) and gave five equivocal results.

Lal et al. (2004) developed a simple and rapid cytometric bead assay that simultaneously quantitated serum IgG antibodies that were directed against the

*Neisseria meningitidis* serogroups, A, C, Y, or W-135. Different bead sets were conjugated with meningococcal capsular polysaccharides A, C, Y, or W-135. A calibrated anti-meningococcal antiserum served as the reference. The tetraplex assay had good intra- and inter-assay variations and showed a strong correlation with ELISAs specific for the same antigens. The authors suggested that assays of this type would be useful to study the efficacy of multivalent vaccines designed to protect against meningococcal infections. Biagini et al. (2003) similarly reported the development of a multiplex bead-based assay that was capable of measuring the antibody response directed against 23 pneumococcal capsular polysaccharides. These pneumococcal polysaccharide serotypes were present in the pneumococcal polysaccharide vaccine (PPV23) licensed by the U.S. Food and Drug Administration.

An indirect multiplexed flow cytometric immunoassay has also been reported that can quantitate specific IgG antibody levels (PE-conjugated anti-human IgG) directed against *Haemophilus influenzae* type b polysaccharide and toxoids from *Clostridium tetani* and *Corynebacterium diphtheriae* (Pickering et al., 2002). These investigators used a pooled serum standard that was calibrated against standard antisera from the World Health Organization (units/mL) and the U.S. Food and Drug Administration ( $\mu\text{g/mL}$ ) for antibody quantitation. The antibody levels measured in pre- and post-vaccination antisera by flow cytometric immunoassay agreed closely with those determined by individual ELISAs. The usefulness of this type of assay for the evaluation of vaccine efficacy was demonstrated.

McHugh et al. (1997) produced a cytometric bead assay for the detection and quantitation of serum antibodies directed against the hepatitis C virus (HCV). Microspheres of different size were coated with proteins from either the viral core or the nonstructural region 3 (NS3). These beads were incubated with serum or plasma samples and developed with PE-conjugated anti-human IgG DAb. The assay mixtures were then analyzed by multiparameter flow cytometric analysis. The forward light-scattering characteristics of the beads were used to identify the smaller (HCV core) and larger (NS3) beads and their associated immunofluorescent (PE) signals. The assay developed by McHugh and colleagues was more sensitive than a commercial ELISA and could resolve indeterminate clinical samples. This type of assay has great potential for screening donor blood samples.

### *Measurement of Microbial Agents*

Multiplex flow cytometric immunoassays have also been developed to identify microbes that are present in specimens. Park et al. (2000) created a competitive inhibition multiplex flow cytometric immunoassay to simultaneously identify 15 *Streptococcus pneumoniae* serotypes. Latex bead sets, of different size and incorporated levels of red fluorescent dye, were coated with different pneumococcal capsular polysaccharides. In brief, the beads were mixed with individual pneumococcal lysates followed by the addition of pooled rabbit antisera against

all serotypes plus fluorescein-conjugated anti-rabbit immunoglobulin DAb. Upon flow cytometric analysis, the fluorescent DAb signals were only inhibited when the homologous serotype of pneumococcus was present. The method was capable of correctly identifying 100% of the pneumococcal isolates tested.

Molecular biological techniques are also compatible with bead-based flow cytometric assays. Different PCR amplification strategies have been used to demonstrate flow methods to measure HIV viral load (Mehrpouyan et al., 1997; Van Cleve et al., 1998). Defoort (2000) developed a multiplex flow cytometric bead-based hybridization assay for the detection of human immunodeficiency virus type 1 (HIV-1) and hepatitis C (HCV) RNA and hepatitis B virus (HBV) DNA using specific oligonucleotide probe-coated, green fluorescent microspheres. The assay was devoid of cross-hybridization between oligonucleotide probes and biotinylated PCR products that were generated by multiplex reverse transcription-PCR. Bound PCR products were detected with PE-cyanin 5-conjugated streptavidin. Methods of this type can be extremely useful for the detection and quantitation of several different pathogens or serotypes in each plasma sample. Flow cytometric assays based on nucleic acid technologies are useful for identifying pathogens not amenable to culture or for when an immunodominant microbial antigen that can serve for serological diagnosis of an ongoing infection has not been characterized. Requirements for highly trained technicians, expensive equipment and materials, and concerns that nucleic acid technologies do not discriminate between viable and dead microbes are potential issues that affect the use of this method.

### *Simultaneous Measurement of Microbes and Toxins*

Novel methods for simultaneously measuring the levels of specific microbes and their products in complex specimens are also possible. As an example, a cytometric bacteria and protein assay (CBPA) was developed (Bolton et al., 2002). For the sandwich flow cytometric immunoassay component of the CBPA, antibodies were generated against *Staphylococcus aureus* enterotoxin B (SEB) and ricin. The antibodies were used to prepare two distinct sets of single-color fluorescent capture beads and PE-conjugated DAb. Purified preparations of SEB and *Ricin communis* agglutinin II served as calibrators. In this case, the identification of bacteria used the capability of the flow cytometer to detect bacteria directly by light scatter or dye-mediated nucleic acid fluorescence. The specific identification was achieved with fluorochrome-labeled species-specific antibodies. The use of membrane permeable and impermeable nucleic acid dyes allowed for the discrimination of live and dead cells. For the simultaneous analysis of killed *Bacillus anthracis* and living *Bacillus subtilis* bacteria, rabbit anti-*B. anthracis* antibodies were conjugated with PE. For the discrimination of total and dead bacteria, the nucleic acid dyes, Syto 59 and propidium iodide were used, respectively.

To perform the CBPA, known amounts of the toxins, bacteria, and capture beads were mixed together in assay diluent along with the PE-conjugated anti-SEB,

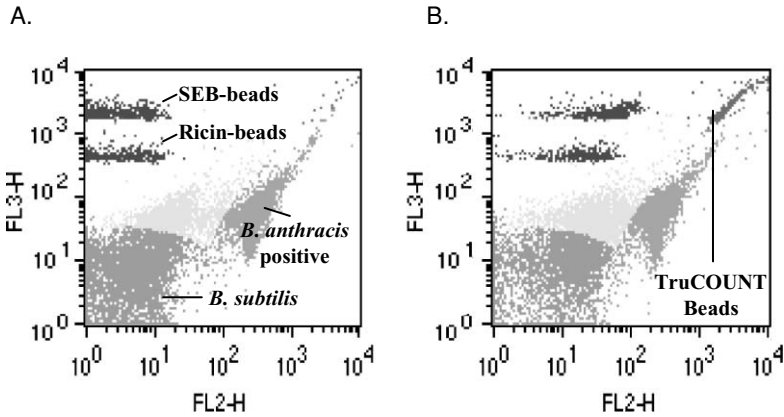


FIGURE 25.2. Analysis of bacteria and toxins by CBPA. Panel A shows the dot plot analysis of two different intensity fluorescent bead populations coupled with antibodies specific for SEB and ricin and two bacteria *B. anthracis* and *B. subtilis*. Dead bacteria (i.e., PI/FL3-positive) that are not labeled with the PE-anti-*B. anthracis* detector antibody appear as light grey dots. Panel B shows the increased FL2 fluorescence of the SEB and ricin beads upon addition of the SEB and ricin toxins and anti-SEB and anti-ricin PE labeled detector antibodies. TruCOUNT beads have also been added to the assay in Panel B.

anti-ricin, and anti-*B. anthracis* antibodies. The mixture was serially diluted and added to TruCount tubes (BD Biosciences, San Jose, CA) for 25 min (room temperature). These tubes contained a known number of fluorescent microspheres to enable the flow cytometric enumeration of bacteria (Alsharif and Godfrey, 2001). PI was added for 30 min followed by the addition of Syto59 (Millard et al., 1997). The mixtures were then analyzed by multicolor flow cytometry with a FACSort (BD Biosciences) cytometer equipped with blue (488 nm) and red (635) lasers to generate the multiparameter fluorescence and forward- and side-light scatter signals.

The dot plots shown in Figure 25.2 (panels A and B) reveal the CBPA's capacity to simultaneously discriminate the bead-based measurement of SEB and ricin toxins level and viable *B. subtilis* and nonviable *B. anthracis*. Figure 25.2 (panel A) shows the dot plot analysis (FL3 vs. FL2) of the single tube assay containing two bacteria, *B. anthracis* and *B. subtilis* stained with Syto59 and PI dyes and two different intensity fluorescent bead populations coupled to capture antibodies specific for SEB and ricin toxins. Panel B shows the result of the assay after adding anti-SEB and anti-ricin PE labeled detector antibodies, which causes an increase in the FL2 fluorescence and shifts the beads to the right and TruCOUNT beads, added to aid in bacterial quantitation. The standard curves for the serially diluted toxins (panel A) and *B. subtilis* counts (panel B) are shown in Fig. 25.3. Bacterial concentrations were calculated from the ratio of counts of bacteria and TruCount microspheres.



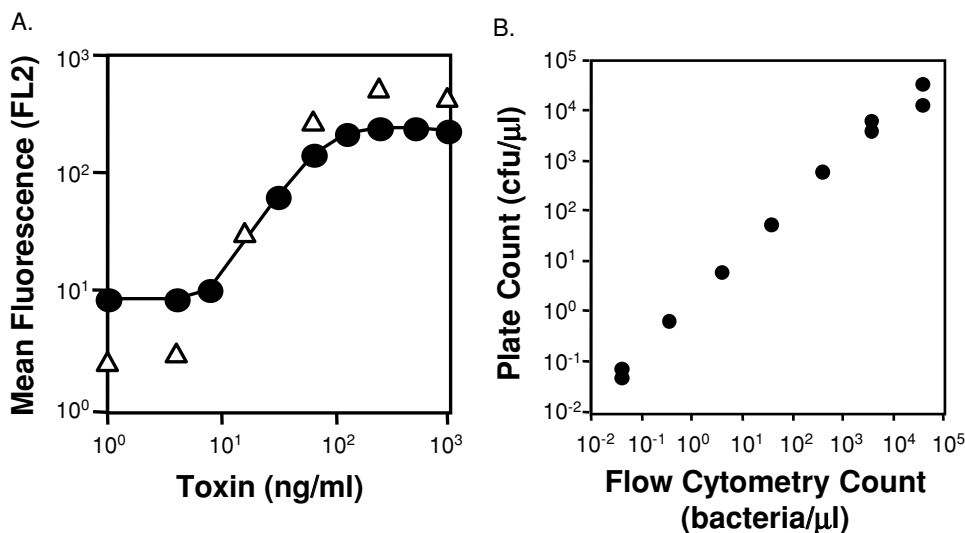


FIGURE 25.3. Standard curves for toxins and viable bacterial counts of *B. subtilis* measured by CBPA. Panel A: Standard curves for toxins: Ricin ( $-\Delta-\Delta-$ ) and SEB ( $-\bullet-\bullet-$ ) proteins were added to the assay at the concentrations indicated and the mean fluorescence intensity (FL2) of the PE-labeled DAb signals were determined for each different protein concentration in a FACSsort. All other components of the assay were present during the titrations. Panel B: Correlation between the concentration of *B. subtilis* determined by flow cytometry and by plating on agar. Serial dilution was made on a *B. subtilis* culture grown in trypticase soy broth. The diluted suspensions were analyzed either by flow cytometry using the nucleic acid dyes plus TruCOUNT beads or were plated on trypticase soy agar to enumerate the bacteria.

## Assessment of Biomarkers Using Multiplexed Bead-Based Assays in Infectious Disease States

### *Cytokine Measurement in Infection and Sepsis*

Numerous investigators (Oliver et al., 1998; Chen et al., 1999), have described multiplex methods to measure panels of secreted cytokines in serum. For example, Carson and Vignali (1999) demonstrated a multiplex bead assay for the simultaneous quantitation of 15 cytokines. The multiplex measurement of cytokine profiles by flow cytometric bead arrays panels may be used to monitor the host response to various infectious disease processes.

Infection and sepsis are a frequent cause of neonatal morbidity and mortality (Tarnok et al., 2003). The early diagnosis of these disease processes is difficult because the clinical signs are highly variable, subtle, and similar to other conditions. Advances in diagnostic detection of pediatric infection and sepsis have been slow, but recent advances in multiplex cytometric bead array analysis of multiple cytokines show promise for improved early diagnoses. Elevated levels of serum

IL-6, IL-8, and IL-10 are among the proposed early indicators of infection and sepsis (Morgan et al., 2004).

Hodge et al. (2005) measured the serum levels of inflammatory cytokines as biomarkers for following the course of pediatric sepsis cases. These investigators used the BD CBA Human Th1/Th2 Cytokine (measures IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$ , and INF- $\gamma$  levels) and Human Inflammatory Cytokine (IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, and TNF- $\alpha$ ) kits for the simultaneous measurement of multiple cytokines in small-volume (0.1 mL) samples. These assays were chosen for their diagnostic potential because of the associated rise in plasma-derived cytokine levels with pediatric sepsis. Cytokines from a group of 18 infected term neonates (culture positive) and a noninfected control group (culture negative) were analyzed. When the cytokine levels of the infected group were compared with the normal ranges of plasma cytokine levels expressed by the control group, all but two patients failed to show significant elevation of the cytokines tested. All other patients showed elevated levels of between one and nine cytokines tested. Although the different cytokine profiles did not correlate with the identity of a specific infectious agent, the authors concluded that these types of assays could be developed to rapidly identify neonates with sepsis.

### *Evaluation of the Immune Pathogenesis of Human SARS Using Multiplexed Bead-Based Flow Cytometric Assays*

Multiplexed bead assays have become very valuable biological tools due to their ability to measure multiple proteins simultaneously in laboratory samples with speed, accuracy, and specificity. Given that the technology is adaptable to the measurement of cytokines, chemokines, or other immune mediators in small volumes of sera or plasma, a role for the multiplex bead assay in profiling the immune pathogenesis or host immune responses involved in human disease has recently emerged.

Severe acute respiratory syndrome (SARS) is a highly contagious respiratory disease caused by the newly identified SARS coronavirus (SARS CoV) that emerged in late 2002 from Guangdong Province, China (CDC Update, 2003; Peiris et al., 2004; Skowronski et al. 2005). SARS has infected more than 8400 persons worldwide to date, killing more than 10% of patients. Those infected with SARS suffer fever, cough, muscle aches, and shortness of breath and often progress to severe lung inflammation. Most patients recover from this illness within 2 weeks, however a large percentage of SARS patients develop severe complications. Although the SARS outbreak of 2003 was largely contained through public health measures, it is clear that the future threat of similar emergent viruses or other microbial agents causing severe respiratory disease will not easily be eliminated.

SARS CoV infection appears to be hallmarked by a poorly defined "cytokine storm" in the lungs and circulatory system of SARS patients (Huang et al.,

2005). Most of the current SARS immunological literature is based on one or a few cytokines, making it unclear how immune responses and progressive lung injury culminates in poor outcomes. In contrast to what one might expect from the immune system when faced with a common microbial invader, it may be that infection and lung injury progresses in SARS patients as a result of uncontrollable innate and adaptive immune responses as reflected by rampant lung inflammation and uncoordinated cytokine production. Because the varying severity of SARS CoV infection appears to be rooted in a patient's own host immune response, SARS represents a unique opportunity to look at pathogenesis of a critical infectious disease and host defense in a new light using multiplexed bead assays, such as BD Biosciences Cytometric Bead Array (CBA) kits.

The public health directive associated with the Toronto SARS outbreak in spring 2003 required all individuals who had contact with SARS-infected patients and who developed any SARS symptoms to present to a hospital for assessment, treatment, and close surveillance. This directive, likely to be a common feature of future outbreaks of emergent or unknown infectious diseases, allowed the collection of specimens within 3 days of onset of symptoms from acutely ill SARS patients as well as a longitudinal study of patients delineated by the severity of SARS disease course (i.e., from mild to moderate symptoms to severe forms of the disease requiring intubation and ICU admittance). CBA results from a representative non-ICU patient shown in Fig. 25.4 shows the high levels of proinflammatory cytokines and chemokines in the plasma of a SARS patient in the early phase (<3 days since onset of symptoms) of disease compared with the convalescent sample and average healthy control levels. As shown, early SARS infection is characterized by increased expression of numerous cytokines and chemokines representing a vigorous acute-phase response (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) as well as strong innate (IL-1 $\beta$ , IL-6, CXCL8, IL-12, and TNF- $\alpha$ ) and IFN- $\gamma$ -driven adaptive immune responses. Levels of expression of these cytokines and chemokines appeared to peak within the first 2 weeks of SARS disease and had generally resolved to healthy control levels at convalescence, probably assisted by aggressive steroid treatment begun at day 7 in this patient.

With elevated levels of IFN- $\gamma$  measurable at the onset of symptoms in the majority of SARS patients, this would suggest that the early skewing of the SARS immunity in favor of a T helper (Th)1-like response during a vigorous early innate immune response may be a critical piece of the puzzle. Because IFN- $\gamma$  and other interferon-response genes are crucial in starting and directing different classes of innate and adaptive immune responses, this would indicate that they also could determine the overall effectiveness, or conversely overall mismanagement, of immune responses against the SARS CoV. Interestingly, other cytokines involved in adaptive immunity and T cell subset regulation, such as IL-2, IL-5, and IL-10, were not expressed at significant levels, although levels of IL-4 did increase slightly during the second week of illness. In other words, to describe SARS as mediated by a generalized "cytokine storm" is probably oversimplified. Instead, the

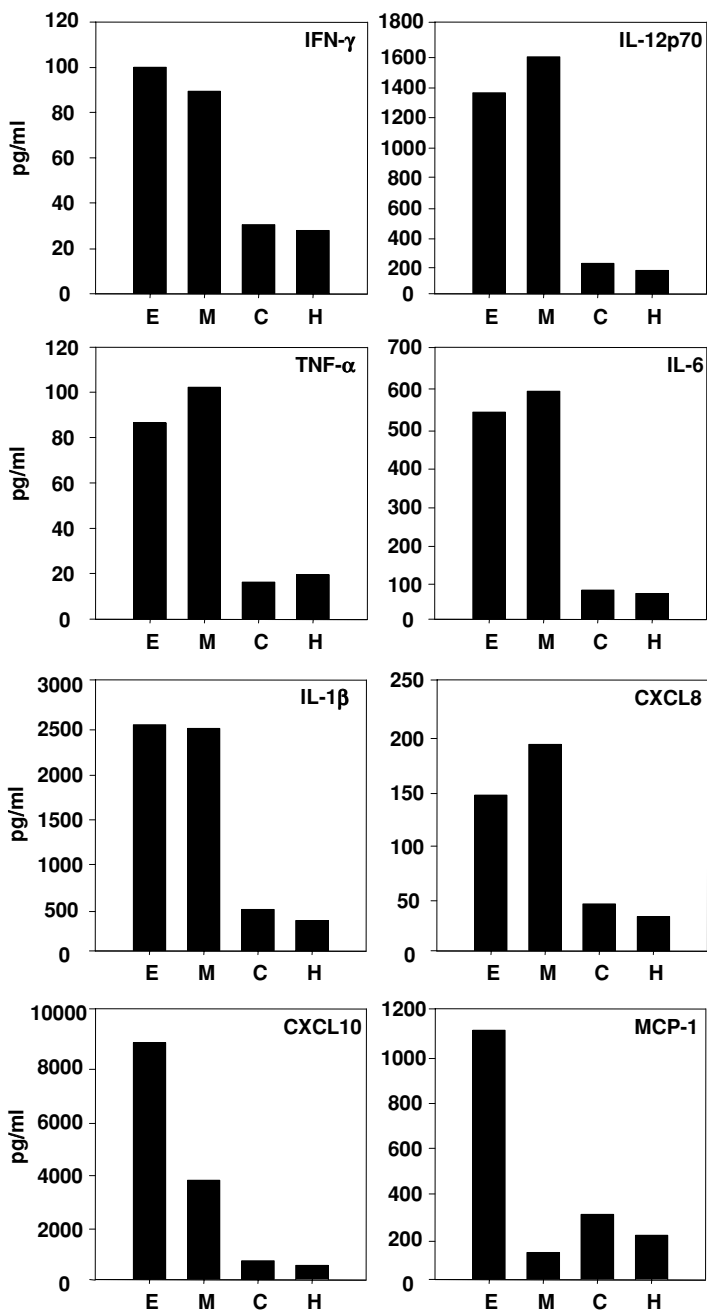


FIGURE 25.4. Plasma concentrations of cytokines and chemokines in a representative SARS CoV–infected patient with a mild to moderate disease course. Cytokine and chemokine levels were measured in 50  $\mu$ L of plasma from one SARS patient within 48 h of onset of symptoms (Early, E), at midpoint (M) of SARS infection (12–14 days), and at convalescence at 30 days (C) by BD Biosciences CBA according to the manufacturer’s instructions. A healthy control average ( $n = 10$ ) is also displayed (H).

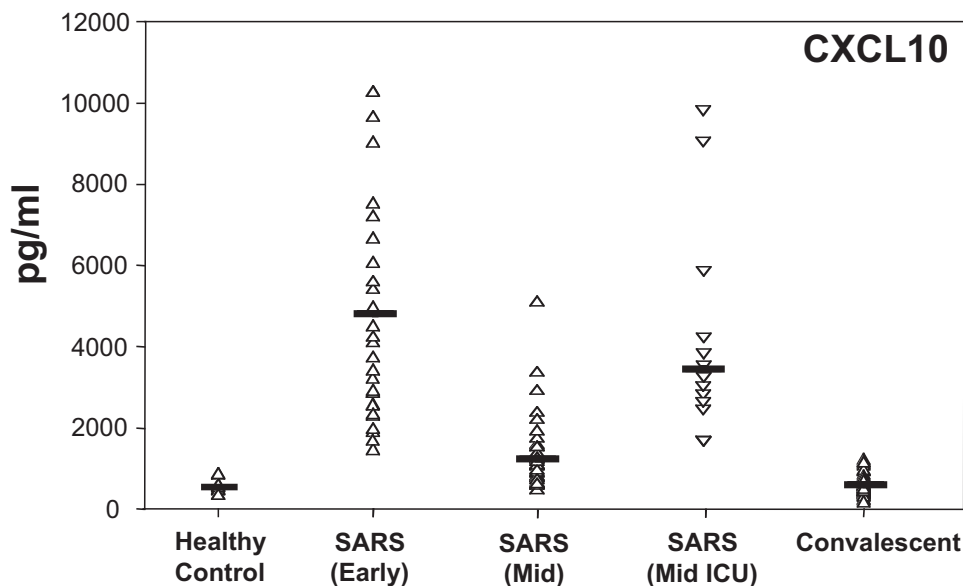


FIGURE 25.5. High plasma concentrations of CXCL10 in SARS patients. CXCL10 levels measured in 50  $\mu$ L of plasma from early SARS patients ( $n = 34$ ) within 48 h of onset of symptoms, 25 SARS patients with mild to moderate symptoms at midpoint of SARS infection (12–14 days), 15 confirmed SARS patients requiring intubation and admittance to the ICU at midpoint of SARS infection (12–14 days), and healthy controls ( $n = 14$ ) by BD Biosciences CBA according to the manufacturer's instructions. Higher levels of CXCL10 were observed in SARS patients relative to controls ( $P < 0.0001$ ). CXCL10 was also significantly lower at 12–14 days in mild to moderate SARS patients compared with those in the ICU ( $P < 0.05$ ).

immunology of SARS appears to be driven over time by a select subset of cytokines and chemokines causing unabated innate and adaptive immune responses in response to the virus.

One chemokine produced at unusually high levels very early in SARS infection is CXCL10 [interferon (IFN)- $\gamma$  inducible protein 10/IP-10]. CXCL10 showed elevated levels in 100% of SARS cases at early onset (Fig. 25.5). In SARS patients with mild to moderate symptoms, CXCL10 levels decrease to near convalescent levels by the mid stage (12–14 days), whereas levels in SARS patients with severe symptoms requiring intubation and admittance to the ICU remain significantly elevated beyond 30 days regardless of aggressive steroid treatment. It is not yet known whether infected tissues are the main source of CXCL10, although the persistence of elevated levels of CXCL10 in patients with severe illness and unresolved SARS CoV infection indicates that this may be the case. Nonetheless, CXCL10 appears to play a unique role in the progress of SARS illness. Chemokines are known for their roles in cell recruitment, but they can also activate immune cells and shape Th1/Th2-type immune responses (Luster, 1998; Rossi and Zlotnik, 2000).

Although interferons and interferon-response genes (i.e., CXCL10) may play a general role in the modulation of respiratory diseases involving lung injury, CXCL10 appears to play a special dual role in SARS. Interferons and CXCL10 responses are necessary to clear SARS CoV if properly timed and modulated but in a minority of patients, CXCL10 participates in the pathogenesis of SARS and poor outcome through uncontrolled inflammation and continued recruitment of activated T cell and mononuclear infiltrates. Indeed, CXCL10 controls the outcome of coronavirus infections in mouse models of other diseases (Liu et al., 2001a, 2001b).

The host immune response to SARS may be the first to be mapped in detail in terms of an emerging infectious disease. Using multiplex bead-based flow cytometric immunoassay technology to study other host responses to infectious diseases may identify novel host response genes that are diagnostic and/or prognostic during the acute phases of the disease. By modeling host gene expression in different subsets of patients from the onset of symptoms through convalescence, multiple genetic pathways may be found that show changes in expression patterns and prognostic capabilities over varying courses of a disease. The role that interferons and interferon response genes play in regulating long-lasting uncontrolled inflammatory events, such as those involved in SARS, is worth careful examination.

In summary, multiplex bead-based flow cytometric immunoassays are emerging as a powerful tool for profiling the nature of microbial agents in biological samples and of host responses in infectious diseases. With the development of new fluorescent dyes, specific high-affinity antibodies, and high-throughput multiparameter methods and flow cytometers, these bead-based immunoassays can have a significant impact on the establishment of prophylactic and therapeutic measures to counter acute and chronic infectious diseases caused by microbial pathogens. With the production of more affordable and user-friendly equipment and materials, people in the developed and developing countries could benefit dramatically from this advanced technique in diagnostic microbiology.

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## References

- Alvarez-Barrientos, A., Arroyo, J., Canton, R., Nombela, C., & Sanchez-Perez, M. (2000). Applications of flow cytometry to clinical microbiology. *Clin Microbiol Rev*, 13, 167–195.
- Alsharif, R., & Godfrey, W. (2001). Bacterial detection and live/dead discrimination by flow cytometry. *BD Biosciences Application Note*, 1–6.

- Andreotti, P. E., Ludwig, G. V., Peruski, A. H., Tuite, J. J., Morse, S. S., & Peruski, L. F., Jr. (2003). Immunoassay of infectious agents. *Biotechniques*, *35*, 850–859.
- Best, L. M., Veldhuijzen van Zanten, S. J., Bezanson, G. S., Haldane, D. J., & Malatjalian, D. A. (1992). Serological detection of *Helicobacter pylori* by a flow microsphere immunofluorescence assay. *J Clin Microbiol*, *30*, 2311–2317.
- Biagini, R. E., Schlottmann, S. A., Sammons, D. L., Smith, J. P., Snawder, J. C., Striley, C. A., MacKenzie, B. A., & Weissman, D. N. (2003). Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. *Clin Diagn Lab Immunol*, *10*, 744–750.
- Bishop, J. E., & Davis, K. A. (1997). A flow cytometric immunoassay for beta2-microglobulin in whole blood. *J Immunol Methods*, *210*, 79–87.
- Bolton, G., Tapia, M., Vegh, F., Alsharif, R., Godfrey, W., & Recktenwald, D. (2002). Multiplexed flow cytometry assay for quantifying bacteria and soluble proteins. Abstract #57827, ISAC Meeting, San Diego, CA.
- Camilla, C., Defoort, J. P., Delaage, M., Auer, R., Quintana, J., Lary, T., Hamelik, R., Prato, S., Casano, B., Martin, M., & Fert, V. (1998). A new flow cytometry-based multi-assay system. 1. Application to cytokine immunoassays. *Cytometry Suppl*, *8*, 132.
- Carson, R. T., & Vignali, D. A. (1999). Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J Immunol Methods*, *227*, 41–52.
- Centers for Disease Control and Prevention. (2003). Update: severe acute respiratory syndrome—worldwide and United States, 2003. *Morb Mortal Weekly Rep*, *52*, 664–665.
- Chen, R., Lowe, L., Wilson, J. D., Crowther, E., Tzeggai, K., Bishop, J. E., & Varro, R. (1999). Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clin Chem*, *45*, 1693–1694.
- Defoort, J. P., Martin, M., Casano, B., Prato, S., Camilla, C., & Fert, V. (2000). Simultaneous detection of multiplex-amplified human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA using a flow cytometer microsphere-based hybridization assay. *J Clin Microbiol*, *38*, 1066–1071.
- Frengen, J., Schmid, R., Kierulf, B., Nustad, K., Paus, E., Berge, A., & Lindmo, T. (1993). Homogeneous immunofluorometric assays of alpha-fetoprotein with macroporous, monosized particles and flow cytometry. *Clin Chem*, *39*, 2174–2181.
- Fulwyler, M. J., McHugh, T. M., Schwadron, R., Scillian, J. J., Lau, D., Busch, M. P., Roy S., & Vyas, G. N. (1988). Immunoreactive bead (IRB) assay for the quantitative and simultaneous flow cytometric detection of multiple soluble analytes. *Cytometry Suppl*, *2*, 19.
- Hodge, G., Hodge, S., Haslam, R., McPhee, A., Sepulveda, H., Morgan, E., Nicholson, I., & Zola, H. (2004). Rapid simultaneous measurement of multiple cytokines using 100 microliter sample volumes—association with neonatal sepsis. *Clin Exp Immunol*, *137*, 402–407.
- Horan, P. K., & Wheelless, L. L. Jr. (1977). Quantitative single cell analysis and sorting. *Science*, *198*, 149–157.
- Huang, K. J., Su, I. J., Theron, M., Wu, Y. C., Lai, S. K., Liu, C. C., & Lei, H. Y. (2005). An interferon-gamma-related cytokine storm in SARS patients. *J Med Virol*, *75*, 185–194.
- Jani, I. V., Janossy, G., Brown, D. W., & Mandy, F. (2002). Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor settings. *Lancet Infect Dis*, *2*, 243–250.

- Lal, G., Balmer, P., Joseph, H., Dawson, M., & Borrow, R. (2004). Development and evaluation of a tetraplex flow cytometric assay for quantitation of serum antibodies to *Neisseria meningitidis* serogroups A, C, Y, and W-135. *Clin Diagn Lab Immunol*, *11*, 272–279.
- Liu, M. T., Chen, B. P., Oertel, P., Buchmeier, M. J., Hamilton, T. A., Armstrong, D. A., & Lane, T. E. (2001a). The CXC chemokines IP-10 and Mig are essential in host defense following infection with a neurotropic coronavirus. *Adv Exp Med Biol*, *494*, 323–327.
- Liu, M. T., Keirstead, H. S., & Lane, T. E. (2001b). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol*, *167*, 4091–4097.
- Luster, A. D. (1998). Chemokines—chemotactic cytokines that mediate inflammation. *N Engl J Med*, *338*, 436–445.
- McHugh TM. (1994). Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods Cell Biol*, *42*, 575–595
- McHugh, T. M., Viele, M. K., Chase, E. S., & Recktenwald, D. J. (1997). The sensitive detection and quantitation of antibody to HCV by using a microsphere-based immunoassay and flow cytometry. *Cytometry*, *29*, 106–12.
- Mehrpouyan, M., Bishop, J. E., Ostrerova, N., Van Cleve, M., & Lohman, K. L. (1997). A rapid and sensitive method for non-isotopic quantitation of HIV-1 RNA using thermophilic SDA and flow cytometry. *Mol Cell Probes*, *11*, 337–347.
- Millard, P. J., Roth, B. L., & Kim, C. H. (1997). Fluorescence-based methods for microbiological characterization of viability assessment. *Biothechnol Int*, *1*, 291–305.
- Morgan, E., Varro, R., Sepulveda, H., Ember, J. A., Apgar, J., Wilson, J., Lowe, L., Chen, R., Shivraj, L., Agadir, A., Campos, R., Ernst, D., & Gaur, A. (2004). Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol*, *110*, 252–266.
- Murray, P. R., & Baron, E. J. (2003). In Murray, P. R., Baron, E. J., et al., eds. *Manual of Clinical Microbiol*, 8th ed. ASM Press, Washington, DC.
- Oliver, K. G., Kettman, J. R., & Fulton, R. J. (1998). Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem*, *44*, 2057–2060.
- Park, M. K., Briles, D. E., & Nahm, M. H. (2000). A latex bead-based flow cytometric immunoassay capable of simultaneous typing of multiple pneumococcal serotypes (Multibead assay). *Clin Diagn Lab Immunol*, *7*, 486–489.
- Peiris, J. S., Guan, Y., & Yuen, K. Y. (2004). Severe acute respiratory syndrome. *Nat Med*, *10*, S88–S97.
- Pickering, J. W., Martins, T. B., Schroder, M. C., & Hill, H. R. (2002). Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for quantitation of antibodies to tetanus, diphtheria, and *Haemophilus influenzae* Type b. *Clin Diagn Lab Immunol*, *9*, 872–876.
- Robertson, B. H., & Nicholson, J. K. A. (2005). New microbiology tools for public health and their implications. *Annu Rev Public Health*, *26*, 281–302.
- Rossi, D., & Zlotnik, A. (2000). The biology of chemokines and their receptors. *Annu Rev Immunol*, *18*, 217–242.
- Skowronski, D. M., Astell, C., Brunham, R. C., Low, D. E., Petric, M., Roper, R. L., Talbot, P. J., Tam, T., & Babiuk, L. (2005). Severe acute respiratory syndrome (SARS): a year in review. *Annu Rev Med*, *56*, 357–381.
- Tarnok, A., Hamsch, J., Chen, R., & Varro, R. (2003). Cytometric bead array to measure six cytokines in twenty-five microliters of serum. *Clin Chem*, *49*, 1000–1002.



- Wild, D., ed. (1994). *The Immunoassay Handbook*. Stockton Press, New York, pp. 906.
- Van Cleve, M., Ostrerova, N., Tietgen, K., Cao, W., Chang, C., Collins, M. L., Kolberg, J., Urdea, M., & Lohman, K. (1998). Direct quantitation of HIV by flow cytometry using branched DNA signal amplification. *Mol Cell Probes*, *12*, 243–247.
- Vignali, D. A. (2000). Multiplexed particle-based flow cytometric assays. *J Immunol Methods*, *243*, 243–255.