

Cytometric bead array: a multiplexed assay platform with applications in various areas of biology

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Abstract

The introduction of flow cytometric bead-based technology has added a new approach for investigators to simultaneously measure multiple analytes in biological and environmental samples. This new technology allows for (1) evaluation of multiple analytes in a single sample; (2) utilization of minimal sample volumes to glean data; (3) reproducibility and results comparative with previous experiments; (4) direct comparison with existing assays; and (5) a more rapid evaluation of multiple samples in a single platform. The cytometric bead array (CBA) system enables simultaneous measurement of multiple analytes in sample volumes too small for traditional immunoassays. Results have been presented for the analysis of a variety of human cytokines. In addition, the technology allows for the design and creation of assays to measure a variety of analytes including inflammatory mediators, chemokines, immunoglobulin isotypes, intracellular signaling molecules, apoptotic mediators, adhesion molecules, and antibodies. New initiatives put forward by the Human Genome Project and the FDA require the development and use of assays for the rapid simultaneous quantitation of multiple analytes. The CBA technology provides the ability to quantify multiple proteins within a given sample, with precision and consistency.

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Introduction to cytometric bead-based assays

The ability to measure soluble mediators involved in immune regulation has been the focus of researchers for over three decades. To this point, immunologists and cell biologists have focused on the *in vivo* and *in vitro* measurement of multiple analytes and their associated receptors. The importance of assessing the relative levels of these mediators is evidenced by the fact that they form the basis of a sophisticated cellular communication network for normal immune function as well as in disease states.

The direct quantification of appropriate analytes, in a timely fashion, is critical for basic researchers and investigators in the pharmaceutical industry. Numerous published methods as well as many different commercial reagents are

available for this purpose. A majority of techniques for the measurement of soluble analytes employ ELISA-based technology. Although this methodology is well suited for single-analyte analysis, it is more desirable to simultaneously quantitate multiple analytes from a relatively small sample size in a rapid fashion. Sample size becomes a critical factor for the evaluation of multiple analytes, especially when monitoring disease states. The melding of ELISA-based technology with flow cytometry has alleviated most of these issues. The utilization of fluorescent-labeled microspheres has been the key to this success.

Flow cytometry may be used as a readout for sandwich immunoassays. Bead-based flow cytometric immunoassays have been described for single analytes including α -feto-protein [1,2] and immune complexes [3]. The single-analyte assay principle was expanded to the detection of multiple analytes from a single sample, using this technology.

Beads of different sizes or colors are used for multiplexed immunoassays. Fulwyler et al. [4] and McHugh [3]

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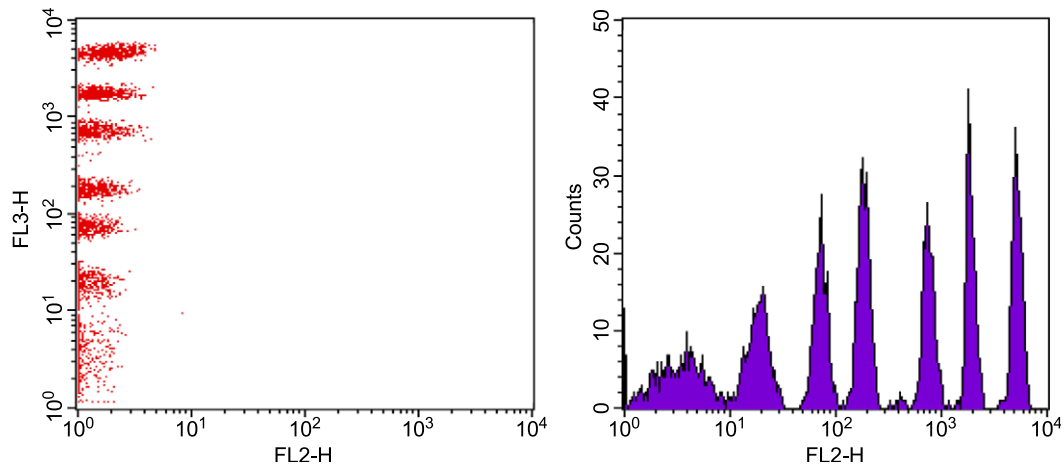


Fig. 1. The bead platform for the CBA system. Particles (7.5 μm in diameter) were internally labeled with different concentrations of a dye that emits strong fluorescent signals measured in the FL3 channel while displaying minimal fluorescence measured in the FL2 channel as shown in the two-color analysis dot plot (left). The nonoverlapping nature of histograms for the bead populations are shown on the right.

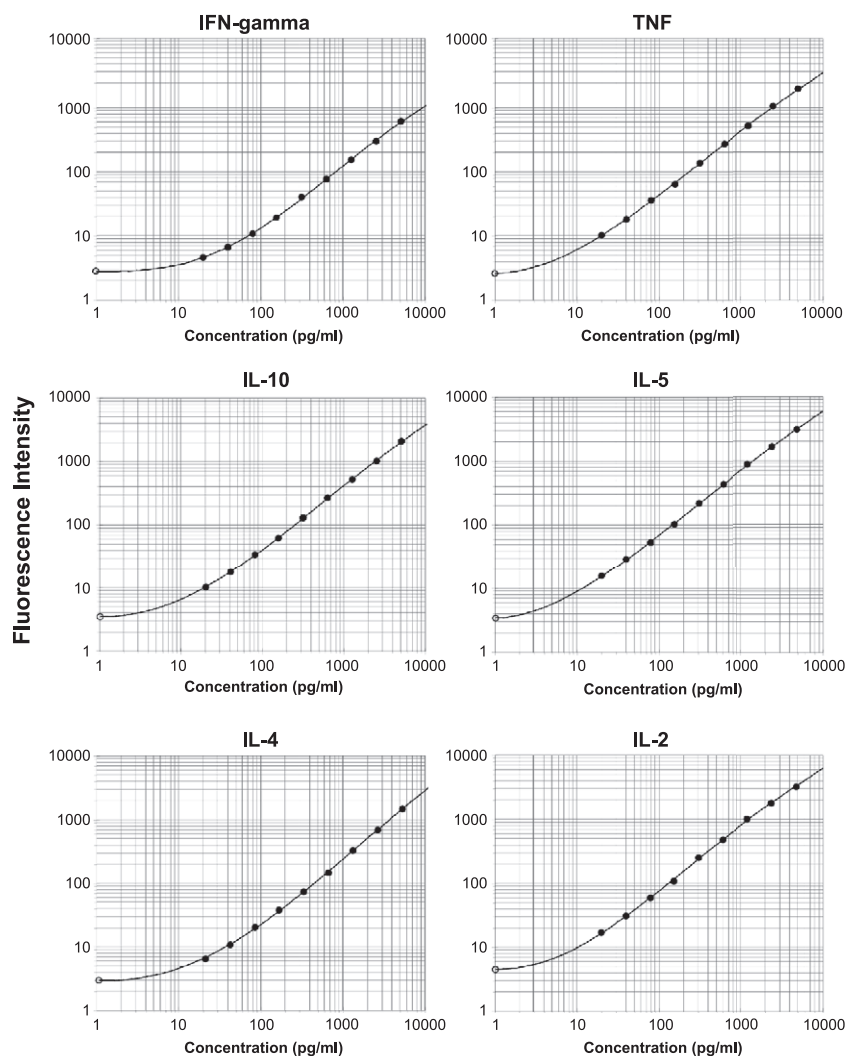


Fig. 2. CBA Software Example of Standard Curves.

used beads of different sizes as carriers for antigens or antibodies. The beads were differentiated by light scatter characteristics and the immunoassay signal was generated through the binding of fluorescent conjugates. Collins et al. [5] used a single bead to measure three different analytes. Three antibodies were coated to the same bead and the corresponding antigens were detected through binding of monoclonal antibodies, each carrying a different fluorochrome. Bead-based technology can also be employed wherein bead populations are identified by one type of fluorescence, while the analyte-dependent signal is generated by detection reagents carrying a second type of fluorescent signal [6]. This concept is useful in creating low-complexity bead sets [7]. Larger sets of distinguishable bead populations can be prepared by staining the beads with two or more fluorescent dyes at various ratios [8]. The immunoassay signal is generated by detection reagents, coupled to a third type of fluorescent dye. Multiplex beads simplify panel assays. Only a single sample is needed to detect and quantify several analytes, an advantage when the sample volume is limited (e.g., pediatric samples, clinical studies, experiments in animal models). Additionally, multiple independent measurements within each bead population assure good precision. The wide detection range (at least three orders of magnitude) for fluorescent signal is another advantage. Bead assays are also well suited to monitor antibody responses against a panel of antigens from infectious agents. McHugh et al. [9] presented a prototypic Hepatitis C virus antibody assay for potential use in the blood bank. Numerous investigators [6,7,10], have described multiplex methods to measure panels of secreted cytokines in serum. For example, Carson and Vignali [11] demonstrated a multiplex bead assay for the simultaneous quantitation of 15 cytokines. In addition, multiplex bead methods have been used to detect antibodies against HLA Class I and Class II antigens [12], autoantibodies to cardiolipin and phosphatidylserine [13], or to assess the phosphorylation levels of proteins [14]. Finally, molecular biology techniques are also compatible with bead assays. Different amplification strategies have been used to develop flow cytometric methods to measure HIV viral load [15,16]. Numerous publications describe the use of multiplex bead method to detect single nucleotide polymorphisms [17–19].

Overall, this technology will (1) allow for the simultaneous measurement of multiple analytes in a quantitative fashion; (2) reduce sample volume for measurement of multiple analytes; and (3) minimize time spent in generating data. In this article, we will focus on a variety of applications of the CBA technology.

Measurement of cytokines

The direct quantitation of cytokines and chemokines from samples has been problematic. We have developed

multiple flow cytometric bead-based immunoassays that are capable of measuring the levels of up to seven different analytes simultaneously, from a single serum, plasma, or cell culture supernatant sample. Particles (7.5 μm in diameter) are dyed with different fluorescence intensities. The dye, incorporated in the beads fluoresces strongly at 650 nm (measured as FL3 signals in BD FACScan and FACScalibur Flow Cytometers) when excited with an argon laser. Each particle population of a given intensity represents a discrete population for constructing an immunoassay for a single analyte (Fig. 1). Each particle is covalently coupled with an antibody (Ab) directed against a specific analyte. These Ab particles, which are unique in their FL3 intensity, serve as capture beads for a specific analyte in the immunoassay panel. When these capture beads for different analytes are used as a mixture, one can simultaneously measure the levels of individual analytes within samples. Detection is mediated by the binding of specific detection antibodies that are directly conjugated with phycoerythrin (PE), to each of the corresponding capture bead/analyte complex populations, thus providing an FL2 fluorescent signal on the appropriate bead. This signal is proportional to the concentration of the analyte in the test matrix. Separately established calibration curves are used to determine the concentration of each analyte in the test sample, using dedicated CBA analysis software.

Assay standardization

A CBA-based immunoassay designed to measure six cytokines commonly associated with Th1/Th2 cellular re-

Table 1
Intra- and interassay precision test of CBA assays

Protein	Intraassay average %CV	Interassay average %CV
IL-1 α	5.7	10
IL-2	3	7.3
IL-4	4	5.3
IL-5	4.7	6.7
IL-6	6.3	9
IL-8	3.7	5
IL-10	2.3	5.7
IL-12p70	3.7	7.3
TNF	4	6.7
IFN- γ	3.7	8
CCL5/RANTES	7.0	10.3
CXCL9/MIG	10.4	11.9
CCL2/MCP-1	6.6	8
CXCL10/IP-10	10.8	10.3

Intra- and interassay reproducibility of the human Th1/Th2 cytokine and chemokine CBA kits. To determine the intraassay reproducibility of the CBA kits, 10 replicate samples of three different levels of the protein standards were tested in a single assay. The average %CV (coefficient of variation) values are depicted in the table (Intraassay Average %CV). In a similar fashion, to determine the interassay reproducibility of the CBA kits, two replicate samples of three different levels of the protein standards were tested in four separate experiments. The average %CV values are depicted in the table (Interassay Average %CV).

Table 2

Conversion factors between the human Th1/Th2 CBA Cytokine Standards and the NIBSC/WHO Standards

	Human IL-2	Human IL-4	Human IL-5	Human IL-6	Human IL-8	Human IL-10	Human TNF	Human IFN- γ	Human RANTES	Human MCP-1
NIBSC code number	86/504	88/656	90/586	89/548	89/520	86/504	87/650	87/586	92/520	92/794
CBA:NIBSC/WHO mass conversion factor	1.52	1.12	0.64	0.86	1.99	1.13	0.76	0.46	1.026	1.05

The relationships of the CBA and NIBSC standard proteins were determined in a parallel titration study. In each case, the NIBSC standard was titrated (starting at 5000 pg/ml) based on its reported nominal mass/vial and tested with the CBA Standards using the CBA assay. The calculated concentrations (mean $\pm$ SEM; $n = 3$) for the NIBSC standards were used to determine conversion factors for standardizing sample concentrations determined with the CBA standards.

sponse was developed. This kit combines beads with the ability to measure the levels of IL-2, IL-4, IL-5, IL-10, TNF, and IFN- γ . The results presented in Fig. 2 represent standard curves with broad dynamic ranges for each of the six cytokines derived from the human Th1/Th2 cytokine kit. A major concern in the utilization of this type of complex assay is the potential variability that may be observed. To address intra- and interassay performance of the human Th1/Th2 cytokine CBA, 10 replicate samples of three different levels of the human Th1/Th2 cytokine standards were tested in a single assay. The average coefficient of variation (CV) values are depicted in Table 1. Coefficient of intraassay variation was found to be less than 6% for all six analytes. To determine interassay reproducibility, two replicate samples of three doses of the human cytokine standards were tested in four separate experiments. Table 1 shows that the CV was less than or equal to 10%. Another concern that arises with the introduction of new technology or methodology is the comparative performance with existing technology. To address this issue, the human Th1/Th2 Cytokine Standards were compared with the National Institutes of Biological Standards and Controls (NIBSC) Standards in

the Th1/Th2 Cytokine CBA. The NIBSC cytokine standards were chosen because they conform to and are recognized by the World Health Organization (WHO) as International Biological Standards. These standards are assigned potency values in International Units (IU) for biological activity and nominal mass values for purposes of bioassay and immunoassay determinations. The results of the comparison of the Th1/Th2 CBA kit Standards and the NIBSC/WHO Standards are summarized in Table 2. The results indicate that the kit can simultaneously measure the levels of six different cytokines with high precision, with a CV typically of less than 10%. The observed parallelism between the dose–response profiles for the protein standards from the human Th1/Th2 cytokine CBA kit and the NIBSC/WHO standards provides confidence in using the assay to reproducibly and accurately measure cytokines in native samples. Moreover, the similar nature of the dose–response slopes for the two sets of standards and native samples provides conversion factors to express CBA determinations of native cytokines in terms of NIBSC/WHO standard concentrations. This makes it possible to determine the equivalency of protein concentrations present in samples measured by different

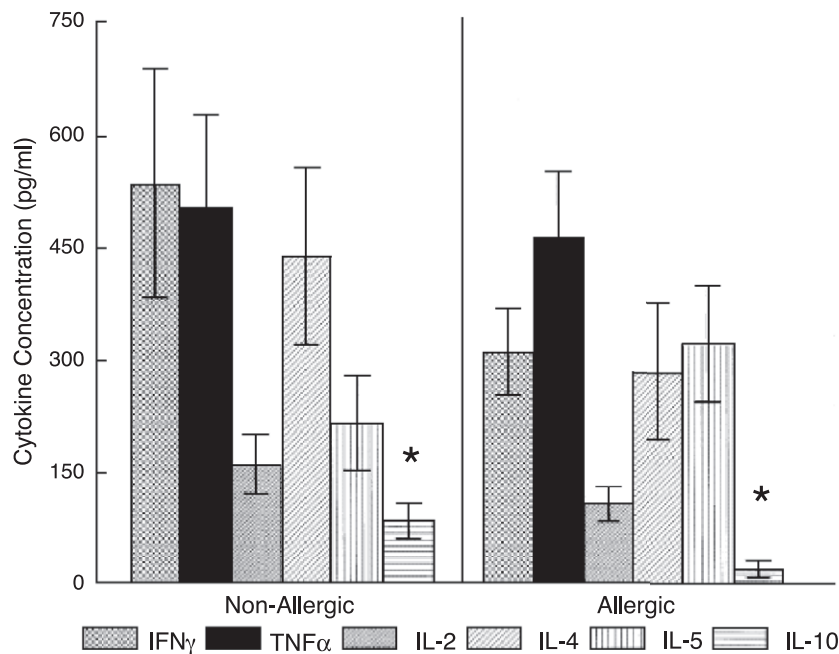


Fig. 3. Concentrations of cytokines measured in nonallergic vs. allergic donor tears.

immunoassays that have been standardized to the same NIBSC/WHO standards.

Cytokine measurement in tear samples

Tears are involved in antimicrobial defense, wound healing, and inflammatory responses [20]. Various ocular disease states cause elevation of inflammatory cytokine levels in tears. However, characterization of tear cytokines has been limited by the small amounts attainable for testing. Tear samples (5–10 μ l) from nonallergic and allergic donors were incubated for 20 h at room temperature with a mixture of capture antibody beads and

detection antibodies. The beads were washed to remove unbound detection antibody and were analyzed using flow cytometry. All six cytokines were detectable in both nonallergic and allergic tears. Tears from allergic donors contained significantly less IL-10 than tears from nonallergic patients. (Fig. 3). The reduced level of IL-10 in the tears of allergic donors could be an important attribute of the pathophysiology of ocular allergic inflammation.

Cytokine measurement in infection and sepsis

Infections and sepsis are among the most common reasons for neonatal morbidity and mortality [21]. The

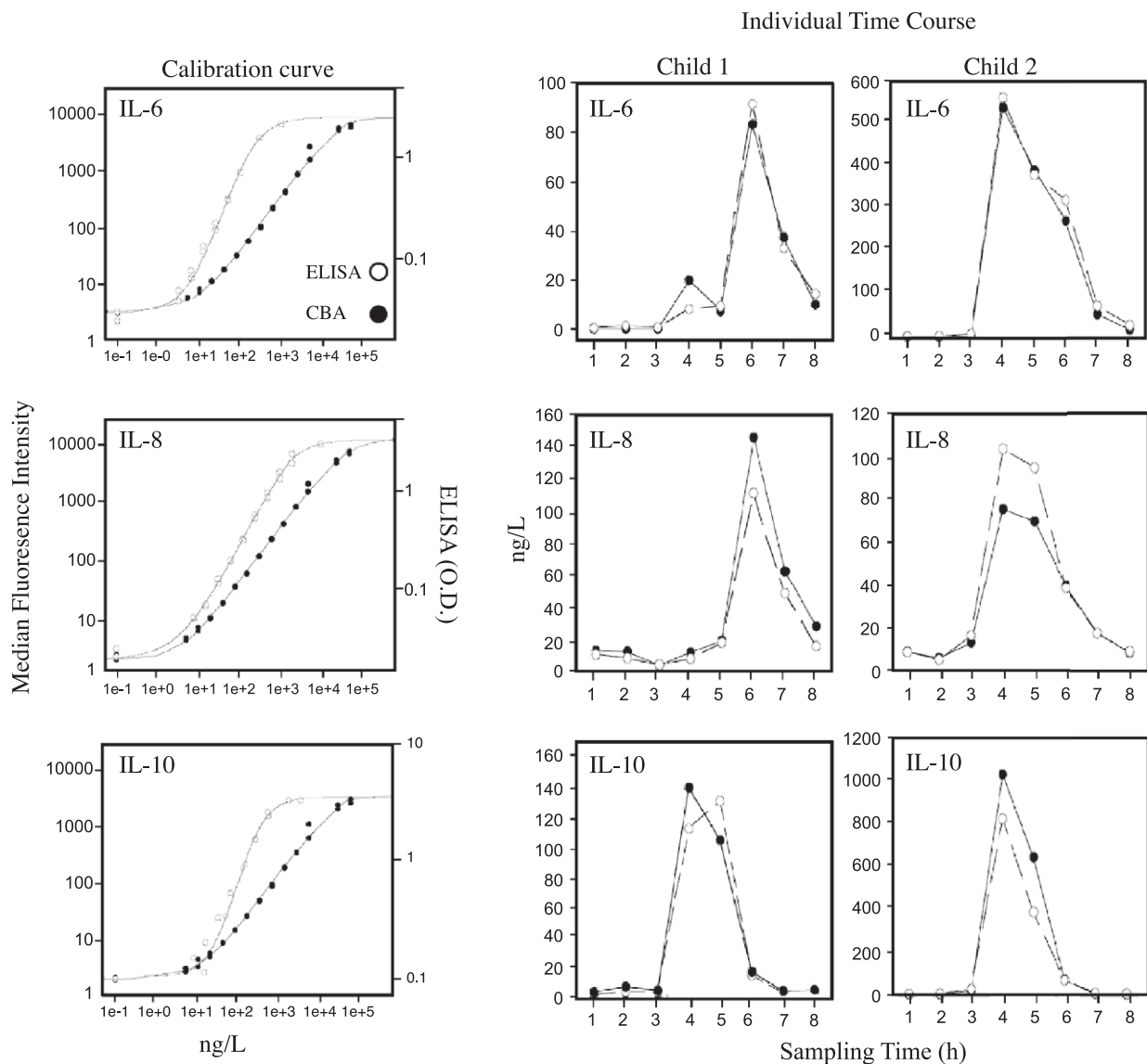


Fig. 4. Serum cytokine levels determined by ELISA and by CBA. The plots show the standard curves (left column). The values for the ELISAs are in absorbance units on the right (y) axis. CBA values are in fluorescence units on the left (y) axis. The individual kinetics of serum cytokines responses (ng/L) in two children undergoing cardiovascular surgery with cardiopulmonary bypass (middle and right columns; Child 1: 11 day old neonate; Child 2: 7.6 year old; x-axis: 1–2 hr: preoperative, 3–5 hr: intraoperative, 6–8 hr: postoperative samples). With ELISA (O) all samples where values exceeded the highest standard were diluted and reanalyzed. With CBA (●) all samples were measured without dilution.

early diagnosis is difficult, because clinical signs are numerous and often subtle. In addition, the signs and symptoms are common to a variety of noninfectious diseases. Among the proposed early indicators of infection and sepsis are serum levels of IL-6, IL-8, and IL-10. The inflammation CBA assay, simultaneously quantifying IL-1 β , IL-6, IL-8, IL-10, IL-12p75, and TNF in a single sample, used 25 μ l of serum sample (Fig. 4). Incubation time was 3 h. CBA beads were analyzed with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with the proprietary software [4]. With the CBA, the dynamic assay range was wider than for all ELISAs by at least an order of magnitude (Fig. 2). The detection limits for IL-6, IL-10, and IL-8 were below 5 pg/ml. The ELISA and CBA values showed good correlation. Quantitative levels of IL-6, IL-8, and IL-10 were virtually identical with both assays as exemplified by the characteristic time course of cytokine production by two patients (Fig. 4).

Cytokine measurement in nasal lavage and sputum

CBA was used to measure several cytokines in nasal lavage and induced sputum samples, collected on days 2, 4, and 7 after Rhinovirus-16 inoculation of mild asthmatic subjects. The CBA technique allowed multiplex measurement of cytokines in a small amount of the airway sample and demonstrated that Rhinovirus-16 infection increases IL-1 β , IL-6 and IL-8 secretion (Fig. 5). The performance of the CBA assays in sputum and nasal lavage, such as sensitivity, recovery, and linearity are comparable to the commercial ELISA assays, but with the benefit of wider detection range, shorter assay time, and requirement of lesser sample volume. Moreover, in asthma patients, the CBA assay successfully detected the peak cytokine responses in induced sputum and nasal lavage 2–4 days after viral inoculation.

Measurement of complement-derived inflammatory mediators

The complement (C) system is a major component of innate immune function. The involvement of complement in the pathogenesis of antigen–antibody-based autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and acute glomerulonephritis is well documented [22–25]. Other pathologies include sepsis [26,27], adult respiratory distress syndrome (ARDS) [28], and multiorgan failure [29]. More recently, activation of the C cascade has been implicated in transplanted organ rejection [30,31] and angioplastic intervention in cardiac disease.

Several proteolytic cleavage products and activation-specific complexes are generated during C activation, such as C3a, C4a, C5a, Bb, sC5b-9. These products reflect C activation, independent of fluctuations in individual complement component levels [32,33]. Anaphylatoxins C3a, C4a, and C5a are bioactive cleavage products released from plasma components C3, C4, and C5 during C activation and are involved in the mediation of various cellular immune responses, as well as being potent pro-inflammatory agents [32,33]. The release of these cleavage products is a reliable indicator of *in vivo* or *in vitro* C activation, and can be used to monitor the clinical status of different immune diseases. The intact anaphylatoxins in serum or plasma are quickly converted into the more stable, less active C3a-desArg, C4a-desArg, or C5a-desArg forms by Carboxypeptidase N. Radioimmunoassay (RIA) or ELISA-type determinations of anaphylatoxins from serum or plasma samples frequently require extra sample preparation steps, such as removal of the precursor molecules by precipitation methods, are time consuming and can lead to under- or overestimation of the analytes. The quantitation of C3a-desArg, C4a-desArg, and C5a-desArg in plasma or experimental samples using neo-epitope-specific monoclonal antibodies avoid these techni-

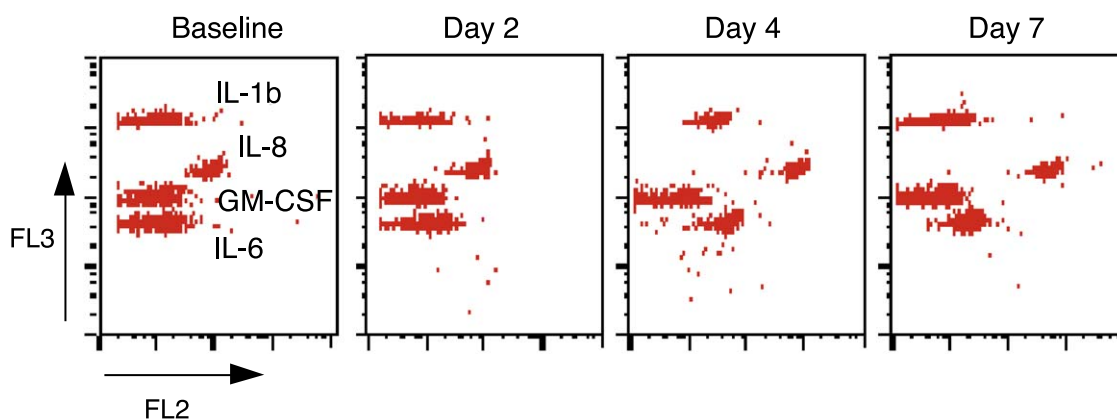


Fig. 5. Cytokine responses in nasal lavage from an asthma patient to Rhinovirus-16 inoculation. Specimens were collected on day 0 (before viral inoculation) and 2, 4, and 7 days after inoculation. A 4-plex cytokine CBA assay, consisting of IL-1 β , IL-8, GM-CSF, and IL-6 beads was used for these samples. Detectable cytokine concentration changes were observed after Rhinovirus-16 inoculation.

cal difficulties and provide a more reliable, faster measurement of the level of complement activation.

Measurement of human C3a-des Arg, C4a-des Arg, and C5a-des Arg

The ability to do a quantitative assessment of these products in serum or plasma has proven to be problematic. A variety of kits are commercially available but most do not address the critical issues important to basic science and pharmaceutical researchers, such as the need for reduced sample size and/or assay times. Depending on the needs of an investigator, multiple approaches may be needed for the appropriate quantitation of a specific analyte. To address these issues, we have employed neopeptide-specific monoclonal antibody technology for easy and accurate detection of C activation products in plasma and other biological fluids. The human anaphylatoxin CBA kit provides a new platform to perform simultaneous, highly sensitive measurements of human anaphylatoxins C3a-desArg, C4a-desArg, and C5a-desArg from a small volume sample. Results from a typical experiments are shown in Fig. 6. The range for C5a-des Arg is 4–1000 pg/ml, while the ranges for C3a-des Arg and C4a-des Arg are 10–2500 pg/ml. A major problem in the quantitative measurement of anaphylatoxins, ex vivo, is the continued activation of the C pathways following sample collection. This problem has often led to erroneous measurement of analyte levels from normal and disease states. A broad spectrum serine–protease inhibitor added to the plasma samples at the time of sample collection provides additional protection from ex vivo C activation, and therefore ensures more accurate measurements. Addition of FUT-

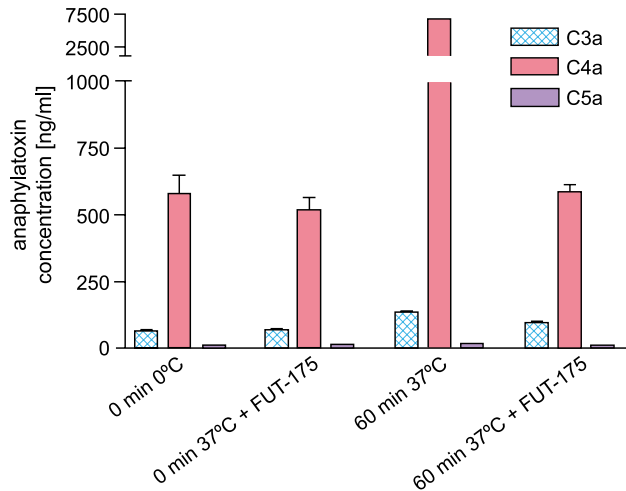
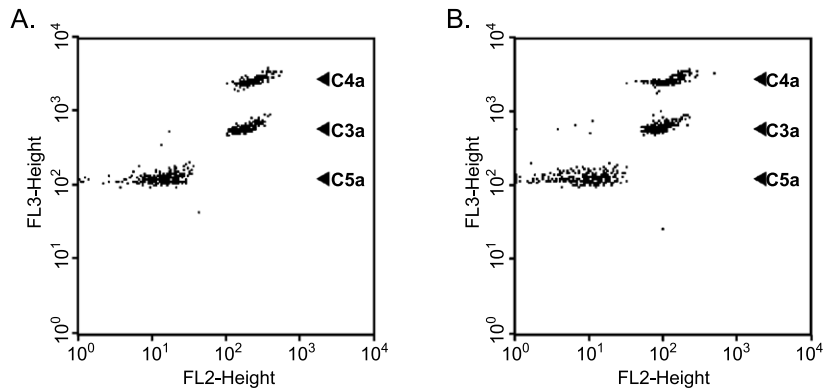


Fig. 7. Normal human EDTA plasma samples with or without 50 µg/ml FUT-175 were incubated at 37°C for 60 min and levels of C3adesArg, C4adesArg, and C5adesArg were analyzed from the same aliquot using human anaphylatoxin CBA kit. Results indicate that after 60-min incubation at 37°C, a moderate elevation of C3adesArg levels and a significant elevation of C4adesArg levels were detected. This is preventable with 50 µg/ml FUT 175.

175 (Futhan-nafamostat mesilate) a protease inhibitor, provides this stabilization [34]. Samples stabilized with FUT-175 reflect the circulating levels of complement activation products at the time of the sample collection. Short-term storage of EDTA plasma samples at elevated temperatures promotes the conversion of C4 resulting in increased levels of C4a-des Arg. The results indicate that C4a-desArg measurements from EDTA plasma samples, collected in the absence or presence of FUT-175, vary widely depending



BD Cytometric Bead Array Analysis

			C5a			C3a			C4a		
SampleID	Acq Date	Dilute Factor	FL2 MFI	Tube pg/ml	Sample pg/ml		Tube pg/ml	Sample pg/ml		Tube pg/ml	Sample pg/ml
NHP #29 800x	18-Mar-02	800	13.9	5.3	4256.3	171.5	78.7	62947.8	218.7	369.7	295747.1
NHP #29 600x	18-Mar-02	1600	9.3	3.2	5151.4	86.6	42.3	67743.6	105.5	176.1	281838.9

Fig. 6. Anaphylatoxin levels were measured from a normal human plasma sample: The same sample was diluted (A) 1:800 and (B) 1:1600. Actual dot plots are shown. Calculated values for this sample indicate 4.70 ng/ml C5adesArg, 65.34 ng/ml C3adesArg, and 288.79 ng/ml C4adesArg. Data acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences).

on temperature of sample storage and the time the sample is analyzed. EDTA-plasma samples collected in FUT-175 containing tubes were stable for 24 h at room temperature. In contrast, samples collected without the inhibitor had a significant elevation in C4a-desArg levels over 24 h. This analysis was conducted with using a C4a-desArg specific ELISA kit (data not shown). To further examine the effectiveness of FUT-175, normal human EDTA-plasma samples were collected in the presence or absence of the inhibitor, incubated at 37°C for 60 min and analyzed for the three anaphylatoxins utilizing the human anaphylatoxin CBA assay (Fig. 7). Dramatic increases in C4a-desArg were observed after 60 min in the absence of FUT-175. In contrast, samples collected in the presence of FUT-175 showed no such elevation in C4a-desArg levels.

Measurement of intracellular signaling molecules

Stimulation of cells through receptors leads to the activation of intracellular signaling pathways, which in turn leads to many different cellular responses. A common feature of signaling pathways, especially in the immune system, is the activation of kinases, which phosphorylate either tyrosine or serine/threonine residues found on a series of other proteins. Many kinases themselves are activated by being phosphorylated. This leads to a signaling cascade or amplification of the response.

T cell activation

A variety of receptors have an intrinsic tyrosine kinase activity while others associate with cytoplasmic tyrosine kinases. An example of this is the activation of T cells through the T cell receptor [35–37]. The receptor itself has no enzymatic activity but it does have ITAM regions, which, upon phosphorylation, serve as docking sites for kinases. Cross-linking of the receptors induces activation of the tyrosine kinase Lck, which phosphorylates the receptor. Another tyrosine kinase, ZAP-70, binds to the phosphorylated ITAM region of the receptor where it is then phosphorylated and activated by Lck. ZAP-70 is responsible for the phosphorylation of a series of adaptor proteins such as LAT and SLP-76, which function as organizing centers for cell signaling. Much of this is occurring in lipid rafts that also serve to segregate and organize signaling proteins. The tyrosine kinase, Itk, is recruited to the adaptors and it is phosphorylated and activated by ZAP-70. Itk and ZAP-70 then phosphorylate phospholipase C, which hydrolyzes polyphosphoinositides to produce diacylglycerol, which activates PKC, and inositol tris-phosphate, which is involved in increasing intracellular calcium. Cytoskeletal changes induced through the activation of Pyk2 and small GTPases help to regulate the T cell response. In addition, the MAP kinase pathway is activated leading to the translocation of various transcription factors to the nucleus followed by the synthesis of new proteins.

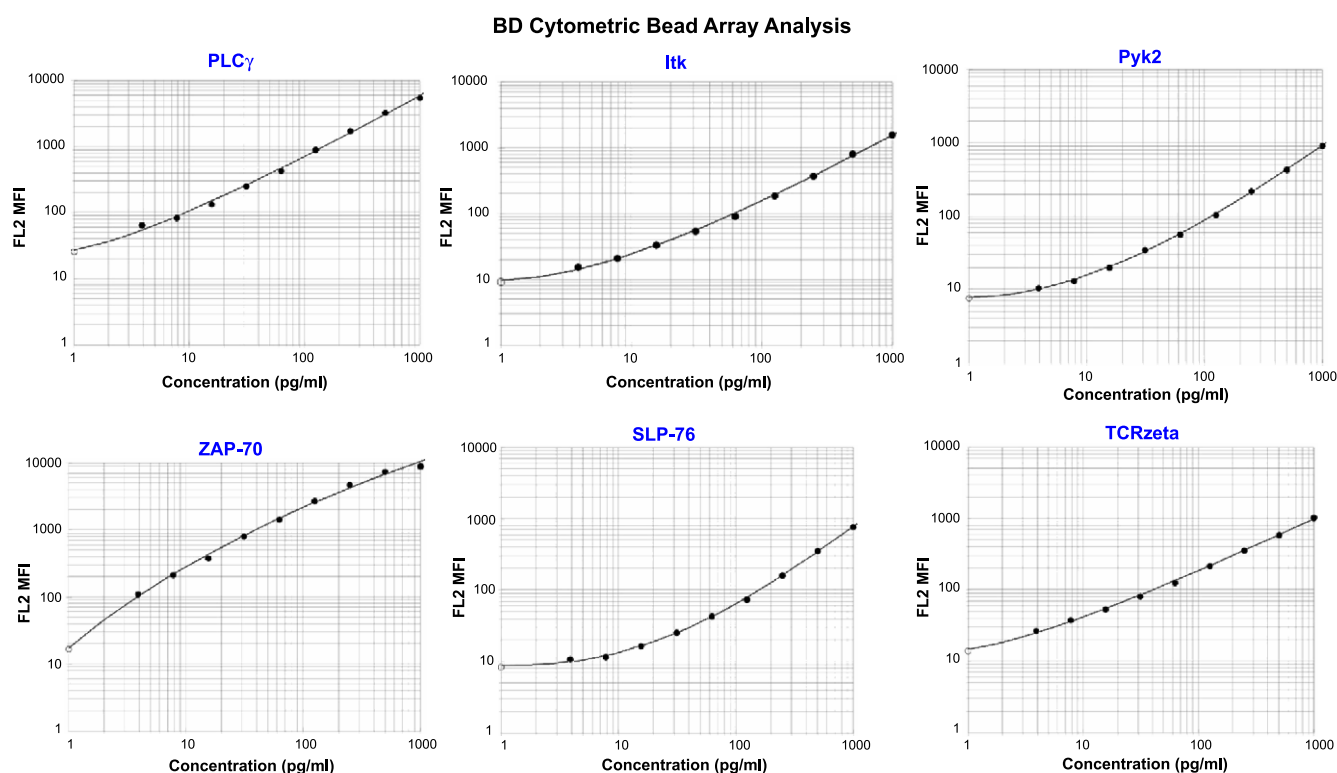


Fig. 8. Human T cell activation CBA standard curves. The human T cell CBA measures phosphorylated phospholipase C, Itk, Pyk2, ZAP 70, SLP 76, and the zeta chain of the T cell receptor. Using an activated lysate, a curve is generated using the CBA software.

Although the most direct method for assessing the activity of a kinase is using an immune complex kinase assay, this involves relatively large amounts of ^{32}P . The most common method is to determine whether the kinase has been phosphorylated itself. Although this is an indirect measure, it works quite well for many kinases. This is often done by immunoprecipitating the kinase followed by a Western blot using an antiphosphotyrosine antibody. With the advent of phosphospecific antibodies, a direct Western blot can now be done. A CBA assay functions in a manner similar to an ELISA. The advantages of this method over Western blots for studying cell signaling are that multiple parameters can be measured using the same sample. In addition, the assay takes less time and the results are quantitative. The human T cell activation CBA measures phosphorylated phospholipase C, Itk, Pyk2, ZAP-70, SLP-76, and the zeta chain of the T cell receptor. These are all upstream or early signaling events. Cells are activated and then lysed and denatured in an SDS containing buffer. Standards are run to generate a standard curve. The top standard is arbitrarily called 1000 units of activity per milliliter. Sample lysates are then run in the assay and the CBA software determines how many units/ml are in the sample. Fig. 8 shows the standard curve for the six different signaling molecules in the human T cell activation CBA assay.

Cytokine and growth factor signaling

Cytokines and growth factors are secreted proteins that are involved in cell growth, differentiation, and activation in the immune system. Early work showed that binding of these factors to their respective receptors causes activation of the Janus kinases (JAK), which are constitutively associated with the receptors [38–41]. The JAKs phosphorylate certain tyrosine residues on the receptor, which act as docking sites for the STAT family of transcription factors. After binding to the receptor, the STATs are also tyrosine phosphorylated by the JAKs. Tyrosine phosphorylation activates the STATs and causes them to dissociate from the receptors. The STATs form either homo- or heterodimers, which are translocated into the nucleus where they bind to DNA and initiate transcription.

Growth factor binding to the EGF receptor is known to activate both JAK1 and JAK2 as well as the intrinsic tyrosine kinase activity of the receptor itself [42]. In addition, members of the src family of tyrosine kinases can also be activated. A variety of substrates are then phosphorylated by these kinases. In addition to the receptor itself, the JAKs and STAT1, 3, and 5 are tyrosine phosphorylated. The adaptor protein Shc also binds to the receptor and is tyrosine phosphorylated [43,44]. This induces binding of the Grb2-SOS complex, activation of small GTPases, and the subsequent activation of the MAP kinase pathway. All of this ultimately leads to cell proliferation.

To determine which signals are essential for cell growth, B82L cells transfected with either human wild type or kinase inactive EGF receptor were tested. In the M721 transfectants, a point mutation at lysine 721, in the ATP binding site, renders the intrinsic kinase inactive. In an earlier study, it was shown that the kinase inactive cells did not exhibit a proliferative response to EGF [45]. EGF causes rapid phosphorylation of STAT1, which peaks in 5 min and then declines over the next 60 min as shown in Fig. 9a. These determinations were made using a CBA assay that measures phosphorylated STAT1. The relative units of activity are based on a calibration curve generated using a phosphorylated, recombinant STAT1 fusion protein as the standard. In contrast, the addition of EGF had almost no effect on the activation of STAT1 in the kinase-inactive mutant cells. This indicates that the kinase activity of the receptor itself is important in EGF-induced STAT1 phosphorylation and not JAK1. The same samples were also analyzed by Western blot using an anti-phospho-STAT1 (Y701), which recognizes phosphorylation at 701 as a comparison (Fig. 9b). As can be seen, there is very good agreement between the results of the CBA analysis and the Western blot. The CBA assay has very good linearity of signal and is sensitive to 0.3

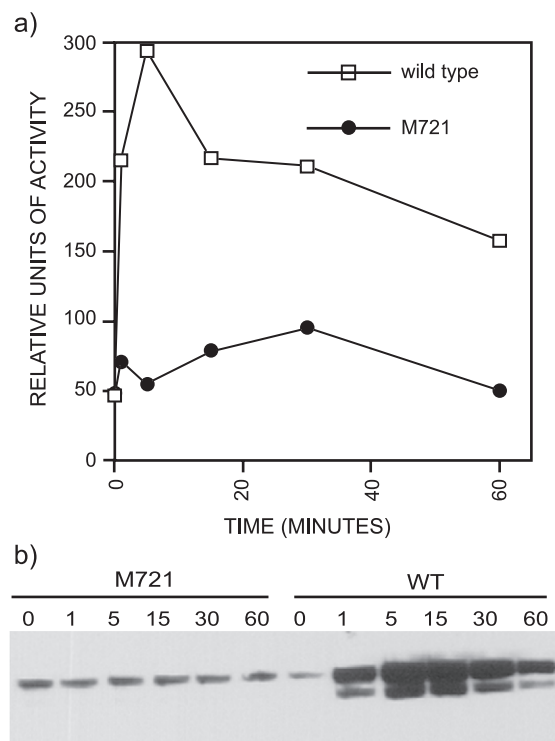


Fig. 9. STAT1 activation in wild type and kinase inactive cells. B82L mouse cells were transfected with either wild type or kinase inactive (M721) human EGF receptor. The cells were serum starved before being activated with EGF for various lengths of time. The cells were lysed by the addition of 1% SDS (final concentration) and placed in a boiling water bath for 5 min. In panel A, the cell lysates were tested in a phosphorylated STAT1 CBA assay. Based on a standard curve, the results are shown as units/ml of phospho-STAT1. Panel B shows the same samples analyzed by Western blot using an anti-phospho-STAT1 (Y701) antibody.

relative units of activity. Both the CBA and the Western blot show similar limits of detection.

These results confirm previous studies on the role of the receptor kinase in growth factor induced cellular activation. Several studies have shown that STAT1 is not activated in cells expressing kinase-inactive receptors [46–48]. It has also been shown that the JAKs are not necessary for STAT1 activation unlike the case with cytokine receptors.

The activation of ERK, JNK, and p38 was also measured using a three-bead CBA that assays phosphorylation of the MAP kinases. Addition of EGF to cells that express the wild-type receptor strongly activated ERK with a peak at 5 min (Fig. 10). JNK and p38

were also activated albeit to a much lesser extent. M721 cells expressing the kinase inactive receptor also showed phosphorylation of all three MAP kinases although less than is seen in the wild type. These results indicate that the kinase activity of the receptor is required, either directly or indirectly, for activation of STAT1 but not of the MAP kinases.

Once again, this confirms previous studies that have shown that ERK is activated even in cells expressing the inactive receptor kinase [45,49]. In addition, the adaptor protein Shc, which couples the receptor to the activation of small GTPases, is also phosphorylated in the mutant cells [48]. The fact that this pathway is still operational might be due to a nonreceptor tyrosine kinase such as src or it may be due to heterodimerization of the kinase inactive receptor with endogenous ErbB2, which does have kinase activity [48,50].

Measurement of apoptosis

Apoptosis, or programmed cell death, is necessary for tissue homeostasis, and its perturbation can lead to multiple disease states [51]. Signaling via apoptosis can generally be divided into receptor- and mitochondrial-mediated pathways. These pathways converge at several downstream points including the mitochondria, caspase activation, and substrate cleavage [52]. When apoptosis is induced, procaspases are proteolytically cleaved and reassembled to form active heterotetrameric enzymes. The process of their activation is believed to be a key event of apoptosis. Active caspase-3 has emerged as a powerful marker of cells undergoing apoptosis and the presence of cleaved or activated caspase-3 is considered to be indicative of apoptosis. In certain cell types, caspase activation is controlled by members of the Bcl-2 family, which include both pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2, Bcl-x) molecules. The cell death regulatory activity of this family appears to depend on their ability to modulate mitochondrial function [53]. The Bcl-2 family members are major regulators of the apoptotic process, whereas caspases are the executioners. The caspases, which are proteases, are known to cleave certain substrates during apoptosis: one characteristic event of apoptosis is the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation [54].

Measurement of active caspase-3, Bcl-2, and cleaved PARP

The impact of apoptosis on cell physiology has driven the development of innovative tools for analysis of this complicated event. We have developed a novel, quantitative, sensitive, and reproducible CBA-based immunoassay. To address whether the CBA-based technology was comparable with classic Western blot analysis, a time course study

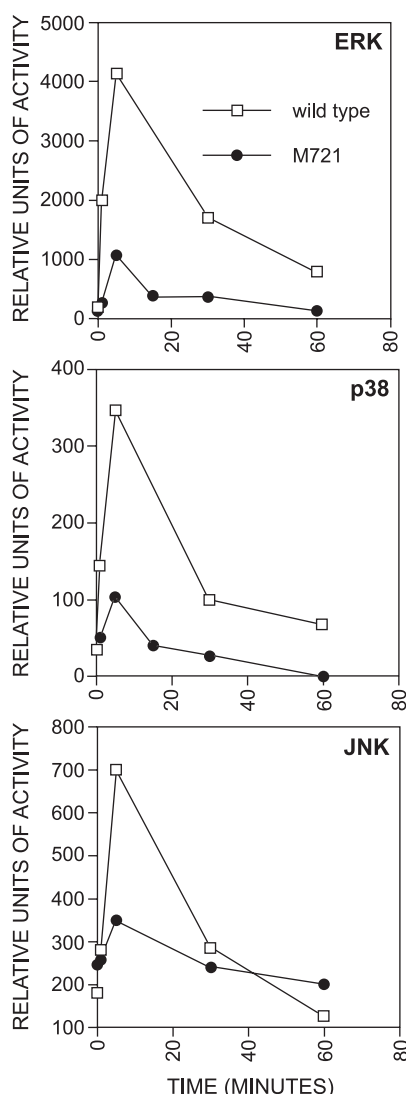


Fig. 10. MAP kinase activation in wild type and kinase inactive cells. Serum-starved cells expressing either wild type or kinase inactive (M721) EGF receptor were activated with EGF for different lengths of time and a denatured cell lysate prepared. The lysates were tested in a MAP kinase CBA assay, which simultaneously measures phospho-ERK, phospho-p38, and phospho-JNK on the same sample. A standard curve was generated and the results are shown in units/ml.

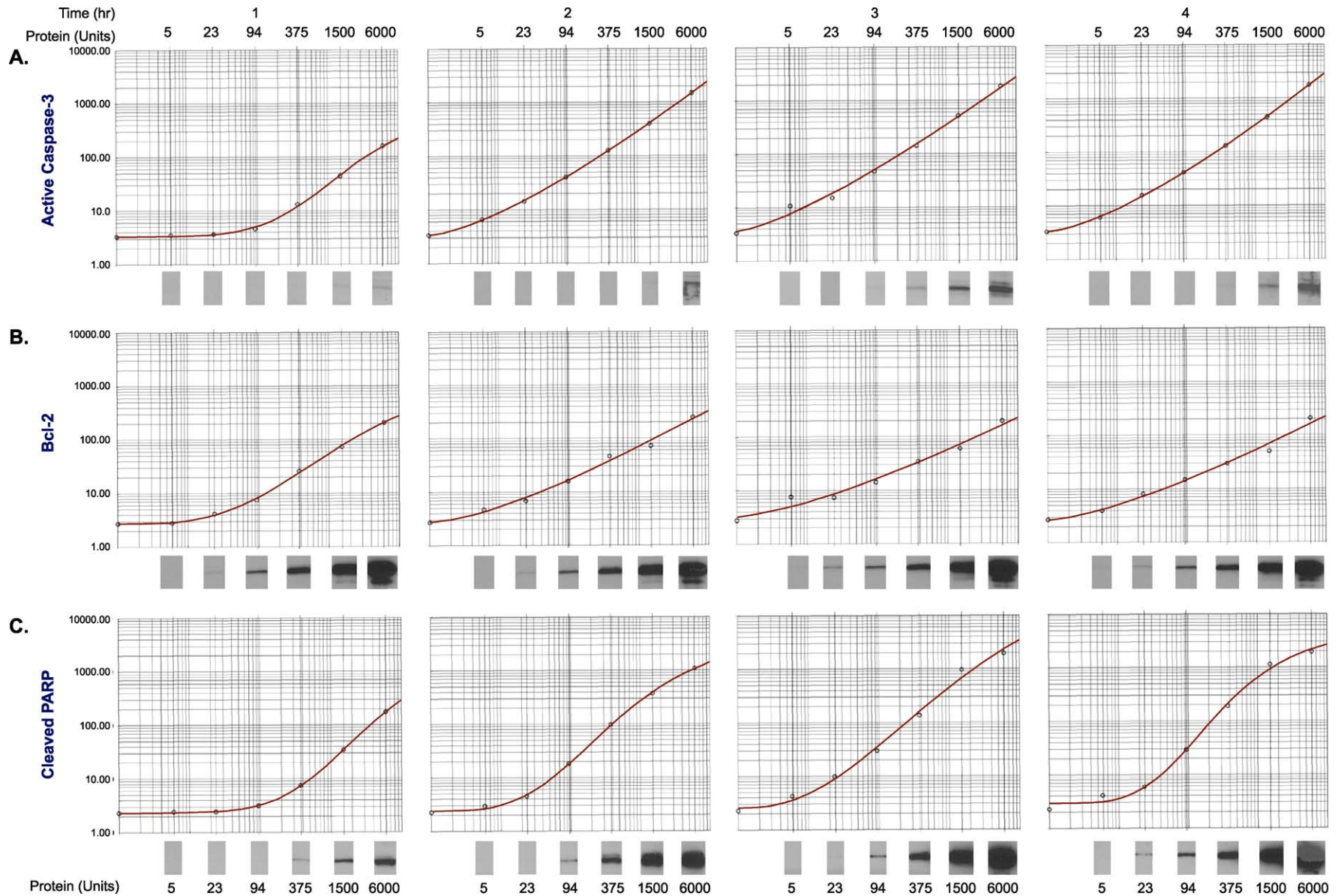


Fig. 11. Time course analysis of active Caspase-3, Bcl-2, and cleaved PARP protein levels in Jurkat T Cells. Cells were treated with 4 μ M camptothecin for 1, 2, 3, or 4 h to induce apoptosis. Cells were washed twice with ice-cold PBS, lysed with lysis buffer, and analyzed for levels of active caspase-3 (A), Bcl-2 (B), and cleaved PARP (C) using the CBA human apoptosis CBA kit. Protein levels are expressed in Units. A unit of active caspase-3, Bcl-2, or cleaved PARP corresponds to the amount of active caspase-3, Bcl-2, or cleaved PARP protein in 0.1 μ g of total protein from camptothecin-treated Jurkat cell lysate. In addition, parallel samples were tested by Western blot (A, B, C). (A) Caspase-3 pAb (poly 1325), (B) Bcl-2 mAb (clone Bcl-2/100), and (C) cleavage specific PARP mAb (clone F21-852) were used for Western blot.

was performed where active Caspase-3, Bcl-2, and cleaved PARP protein levels from camptothecin treated Jurkat T cells were analyzed from a single sample of cell lysate (Fig. 11). The analyses indicate that the CBA assay is capable of measuring these proteins over a two-log scale dynamic range. The standard lysate was tested from 5 to 6000 units for each protein.

A unit of active caspase-3, Bcl-2, or cleaved PARP corresponded to the amount of these analytes in 0.1 μ g of total protein from camptothecin-treated Jurkat cell lysates using a 1-h protocol, thus providing a significant improvement over conventional Western blot assays. To further assess the capabilities of the CBA format, active caspase-3, Bcl-2, and cleaved PARP levels were measured in lysates derived from control and camptothecin treated U937 and MCF-7 cells. The results indicate that both active caspase-3 and cleaved PARP are upregulated in lysates derived from camptothecin-treated U937 cells. In contrast, lysates derived from MCF-7 cells demonstrated an upregulation of cleaved PARP but not active caspase-3 (Fig. 12). This finding was not unexpected considering that the MCF-7 cell line lack the expression of caspase-3 due to a mutation in the caspase-3 gene. In both cell lines, Bcl-2 remained unchanged after camptothecin treatment. In summary, this modality of analysis will provide researchers with new semiquantitative assays to dissect these complicated pathways.

Detection and quantitation of specific antibodies

The bead-based assay system can be easily adapted to carry out immunoassays for detecting antibodies to various antigens either in serum or culture supernatants. This can be accomplished by following variations of assay design as described for other analytes. In one approach, antigens that are amenable to immobilization on solid surfaces could be conjugated covalently or in a noncovalent manner to the beads. Incubation of antigen-coated beads with any biological sample of interest, serum, CSF, or culture supernatants is followed by incubation with species-specific anti-immunoglobulin reagent (e.g., antimouse Ig, antihuman Ig) labeled with a fluorochrome (PE, FITC). The shift in the mean fluorescence intensity (MFI) in the channel reading the detector fluorochrome would reflect the amount of antibody bound to the antigen on the bead. Comparison with a calibration curve created with known amounts of antibody would allow quantitation of specific antibody using the CBA assay. In a variation of assay design, antigen could be immobilized on the beads through a ‘bridging’ or ‘orienting’ intermediate. An epitope-specific antibody is first conjugated to the bead and used to orient the antigen on the bead. Subsequent steps remain essentially the same as described above. However, selection of the anti-species-Ig specific reagent would require eliminating such detector reagents that

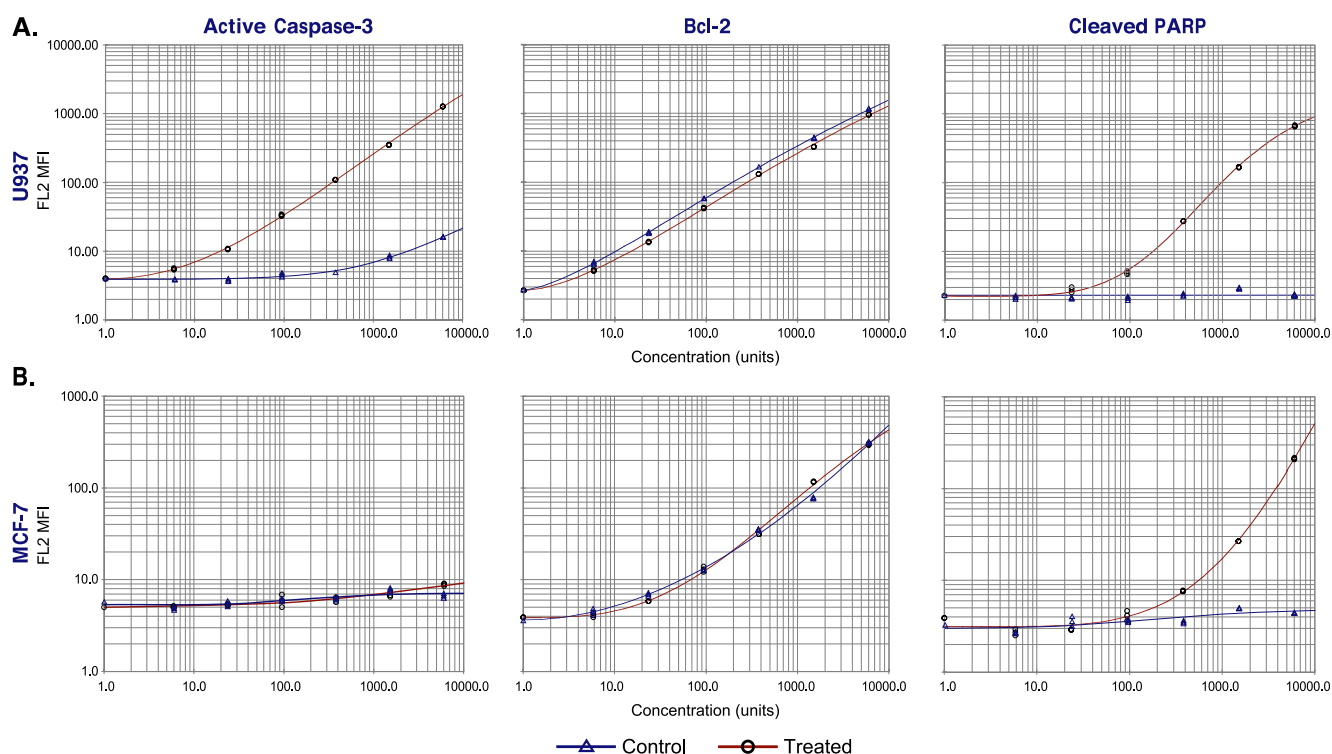


Fig. 12. Active Caspase-3, Bcl-2, and cleaved PARP levels in U937 and MCF-7 Cells. U937 (A) and MCF-7 (B) cells were either untreated (control) or treated with 4 μ M camptothecin for 4 h to induce apoptosis. Cells were washed twice with ice-cold PBS, lysed with lysis buffer, and analyzed for levels of active caspase-3, Bcl-2, and cleaved PARP using a novel CBA apoptosis kit. Protein levels are expressed in Units.

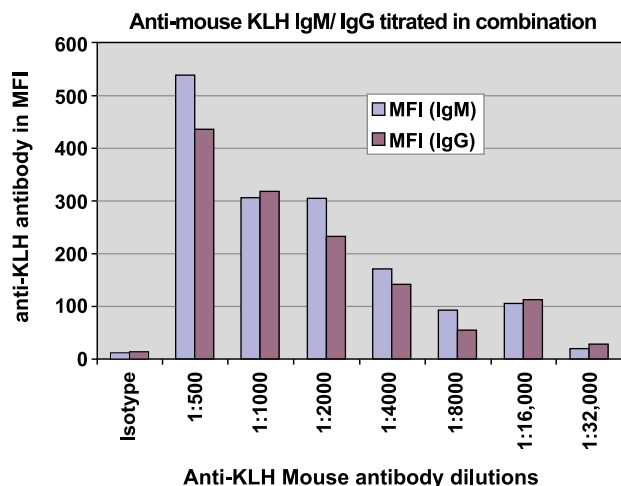


Fig. 13. CBA for measuring the levels of mouse antibodies specific for keyhole limpet hemocyanin (KLH). CBA beads were conjugated with the KLH antigen. Purified mouse IgG₃ and IgM anti-KLH monoclonal antibodies were mixed 1:1 (starting concentration at 1:500 dilution is 0.5 μ g/ml for each antibody). Serial doubling dilutions of the antibody mixture (50 μ l) were mixed with KLH-beads (50 μ l) in tubes. After 1-h incubation, the beads were washed and phycoerythrin-labeled antimouse IgM and fluorescein-labeled antimouse IgG₃ were mixed 1:1 and added (0.1 μ g of each secondary antibody in 50 μ l) to the reaction tubes. The mean fluorescence intensities (MFI) for the IgG₃ and IgM anti-KLH antibodies are shown here. Purified monoclonal mouse IgG₃ and IgM with no specificity for KLH were used as background immunoglobulin isotype controls.

may react or cross-react with the capture antibody on the bead resulting in false positives.

The cytometric bead-based assay allows profiling of humoral immune response using relatively small amounts of sample. Multiple epitopes of the same antigen or multiple antigens of interest (six to eight) could be immobilized on the beads of varying but defined fluorescence intensities. Incubation of multiple beads at once with the serum sample of interest would yield information on the level of specific antibodies to different antigens or epitopes.

In a variation, we designed an assay to detect IgG and IgM response to a model antigen. KLH-conjugated beads were incubated with sera from mice immunized with KLH and the presence of anti-KLH IgG and anti-KLH IgM was revealed by incubating with mouse IgM- and IgG-specific reagents labeled with FITC and PE, respectively. Fig. 13 shows the results of one such experiment where serum from an immunized mouse was tested at various dilutions. The bead-based assay allowed simultaneous detection of the two subclasses of immunoglobulins specific for KLH from one sample at each dilution.

Multiplex bead-based immunoassay data analysis

Bead-based immunoassays such as the CBA are powerful tools to measure multiple analytes (e.g., proteins and mRNAs) within individual samples. Multiparameter analysis of the large amounts of data generated by bead-based assays

provides challenges to the investigator. This data must be quickly and efficiently reanalyzed and converted from a general list of characteristics about the sample to specific, useful, and comparable results for individual analytes. Without a rapid and simple system to reanalyze archived data files to extract the information about each bead population, all advantages of the bead-based assay's multiplex capabilities would be lost.

The data collected using a flow cytometer include information about the physical and spectral parameters of the beads such as size and the fluorescence emission characteristics of each bead population. These fluorescence emission characteristics include the fluorescent emission of the dyed beads (Fig. 1) and the potential fluorescent emissions of the detection fluorochrome (e.g., Phycoerythrin). When samples are analyzed using a flow cytometer in conjunction with a typical data acquisition and analysis package (e.g., BD CellQuest™ software), a list-mode data file is saved using a flow cytometry standard file format, FCS. The data stored in the FCS files can be reanalyzed to determine the median fluorescence intensities (MFI) of the various bead populations, defined by their unique physical and spectral characteristics, to then compare reference samples with unknowns. Analyte levels within individual samples can then be calculated from calibration curves generated by serial dilutions of standard analyte solutions of known concentration. Multiparameter flow cytometric analysis of multiple bead populations is significantly more cumbersome than analysis of individual parameters. An automated or semiautomated analysis method is necessary for rapid reanalysis of the data stored in each FCS file. For the CBA assays, this analysis method is a unique software package, the BD CBA Software. The BD CBA Software is written in the Microsoft® Excel Visual Basic for Applications (VBA) programming language. The CBA Software, in its most recent versions, recognizes FCS 2.0 and 3.0 format data files and automates the identification of CBA bead populations and the determination of detector fluorochrome MFI values for each bead population within the data file for a single sample. Using this data analysis function of the CBA Software for multiple standard files, the MFI values for standards can be determined and plotted. From the plotted standard curve and complex mathematical interpolation, values for unknown samples can be rapidly determined in comparison to known standards using the CBA Software.

For clinically relevant studies, data integrity is of critical importance. Currently, the CBA Software does not include stringent electronic record-keeping capabilities required for FDA Rule 21 CFR Part 11 compliance. It is therefore necessary to utilize the CBA Software in conjunction with other data management systems¹ for clinical trials or other

¹ The BD CBA Software is compatible with the NuGenesis® Scientific Data Management System. More information is available at the NuGenesis website: http://www.nugenesis.com/pages/news/press-releases/en/2002/pr2002_013.html.

experiments that must be managed in compliance with existing FDA requirements. This is important when considering the impact that multiplex assays can have in drug discovery and clinical investigations.

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