Analyte and label binding assay read by flow cytometry

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A new immunometric two-site sandwich assay is introduced, in which a label-scavenging binding partner is added to the sample in addition to the analyte-binding partner. The scavenger binding partner binds excess label antibody, giving a signal proportional to the amount of excess label antibody in the sample solution. A set of two calibration curves is obtained from the two binding partners simultaneously, and a combination of the two signals gives an unambiguous determination of the analyte concentration, even for high analyte concentrations where the hook effect may occur. Two-particle immunofluorometric assays developed for placental alkaline phosphatase and human chorionic gonadotropin on the basis of this principle and yielding signals measured by flow cytometry gave rapid results (2 h) and had working ranges in excess of 5 and 6 orders of magnitude for the respective analytes.

INDEXING TERMS: human chorionic gonadotropin • immunofluorometric assay • monoclonal antibodies • alkaline phosphatase

One-step sandwich immunoassays, i.e., assays in which the analyte and the label antibody are added without an in-between washing step, are gradually replacing the traditional two-step immunoassays, given the advantages of faster assays and simpler implementation [1]. However, all one-step sandwich immunoassays are subject to the hook effect, i.e., a decreasing signal for analyte concentrations that exceed the binding capacity of the signal and capture antibody [1]. The hook effect was first observed by Miles et al. [2] in a two-step immunoassay, but a more pronounced effect can generally be observed in one-step immunoassays, as reported by Nomura et al. [3]. The effect has generally been considered undesirable, because two different analyte concentrations may produce the same (indistinguishable) result. To overcome this problem, samples are frequently analyzed in two different dilutions, providing a stronger response for the diluted sample if the hook effect has occurred. Hoffman et al. [4], moreover, developed an alternative “kinetic rate monitoring IRMA system” to identify the hook effect, in which the sample is analyzed after different incubation times before equilibrium. Both of these procedures require an additional analysis step, which complicates the assay.

Flow cytometry is a technique for analyzing cells or particles according to their size and the amount of fluorescence bound to them. As proposed by Fulwyler [5], this technique can utilize particles of different sizes with binding partners of different specificity for quantifying multiple analytes in the same sample. In particle-based immunoassays read by flow cytometry, monosized particles pass one at a time through a focused excitation light beam. The light excites the fluorescence marker specifically bound to each particle. By detecting the emitted fluorescence light pulse and converting it to an electric pulse, one can analyze the individual intensities of a large number of particles and classify these into histograms to obtain an accurate distribution and measure of the mean fluorescence signal from each population or type of particle. In flow-cytometric assays, the hook effect can be identified without additional analysis through simultaneous independent determinations of immunofluorescence from particles of different size. To obtain the additional information required to identify the hook effect, two alternative flow-cytometric approaches relying on this principle have been proposed [6, 7].

In the present study, we evaluated a new variant of flow-cytometric multiobservable assays, based on an assay design proposed by Frengen [8]. These assays involve the use of two particle types—one (MPa) being coated with antibodies directed against the analyte, as in a conventional assay, and the other (MPb) being coated with the analyte itself or with its label-binding part (Fig. 1). Alternatively, this second type of particle may be coated with an antidiotypic antibody directed against the binding site of the label antibody. The MPb particles act as a scavenger and will bind excess label antibody not complexed with the analyte; meanwhile, the ordinary MPa particles bind the analyte, as usual.

Simultaneous measurements of the signals from the two

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Materials and Methods

Monoclonal antibodies (mAbs). The mouse mAbs E26 (IgG1) and E27 (IgG2a) [9] are directed against epitopes on the α- and β-subunits of hCG, respectively. E26 was used as capture antibody and E27 was used as label antibody. These mAbs were kindly provided by Elisabeth Paus of The Central Laboratory at The Norwegian Radium Hospital, Oslo, Norway.

Mouse mAbs F6 (IgG2a), H7 (IgG2a), and α-H7 (IgG1) [10, 11] (InRo Biomedtek AB, Umeå, Sweden) were used in the PLAP assay. The F6 and H7 mAbs, having specificity for different PLAP epitopes, were used as capture and label antibody, respectively; α-H7 is an antiidiotypic mAb directed against the antigen-binding site of H7.

Immunofluorometric assay (IFMA) diluent. Phosphate-buffered saline (cat. no. P-4417; Sigma Chemical Co., St. Louis, MO) was supplemented with (per liter) 10 g of bovine serum albumin (cat. no. A-2153; Sigma), 1 g of NaCl, and 1 mL of Tween 20 (Merck, Darmstadt, Germany).

Preparative procedures

Antibody biotinylation. mAbs E27 and H7 were labeled with biotin according to the guidelines given by Bayer and Wilchek [13] and with use of reagents and assay protocols as previously described [9, 14]. In short, an N-hydroxysuccinimide derivatize of biotin (NHS-Long-Arm-Biotin; Vector Labs., Burlingame, CA), freshly dissolved in 10 μL of dimethyl sulfoxide, was added in 10× molar excess to the mAbs in pH 8.0 borate buffer and incubated on a horizontal rotational shaker for 16–20 h at 4 °C. The labeled antibody was separated from excess NHS-Long-Arm-biotin by membrane dialysis (Spectra/Por Membrane, MWCO 6000–8000; Spectrum Medical Industries, Houston, TX) and passed through a 0.2-μm pore-size filter (cat. no. DG2 M-330-50S; Microgon, Laguna Hills, CA) before storage in IFMA diluent at 4 °C.

Microsphere particles. To set up the ALBA, we used monodisperse macroporous acrylate particles (nominal diameters, 6.5 and 7.5 μm) developed at SINTEF (Trondheim, Norway) for flow-cytometric assays [14] by using the activated swelling method developed by Ugeland et al. [15, 16]. The surface epoxy groups of the particles provided covalent attachment of antibody. To monitor the fluorescence intensity and normalize the signal, we used 2.2-μm-diameter compact fluorescent polystyrene particles (cat. no. 09847; Polyscience, Warrington, PA).

Particle coating. mAbs E26, F6, and α-H7 were coated onto macroporous particles of 6.5-, 7.5-, and 6.5-μm diameter,
respectively, as described earlier [14], 150 μg of antibody per 1 mg of particles. About 80% of the antibody added was permanently bound to the particles. Macroporous particles of 7.5-μm diameter were coated similarly with purified hCG β-subunit (Calbiochem; cat. no. 969126), 30 μg/mg particles. Analyte-binding particles coated with either E26 or F6 were the MPa particles; the particles coated with either β-hCG or α-H7 were the MPb scavenger particles (Fig. 1).

**ALBA PROCEDURE**

In the PLAP assay, 10 volumes of 0.9 mg/L biotinylated H7 was incubated with 1 volume of streptavidin–R-phycocerythrin (SAP-PE, cat. no. 9023; Becton Dickinson, San Jose, CA) for at least 15 min before use. The amount of labeling and the concentration of the label antibody in the PLAP assay were chosen to optimize the assay signal-to-noise ratio (results not shown). For the hCG assay, 1.9 mg/L biotinylated E27 was incubated with SAP-PE (6:1 by vol) for at least 15 min before use. The amount of labeling and the concentration of the label antibody were the same as in previous optimizations for the hCG assay [9].

In each of the two series (PLAP and hCG) of assay reagent tubes, 20 μL of labeled antibody and 50 μL of IFMA diluted containing intensity monitoring particles (~6000 particles per milliliter) were mixed with 10 μL of analyte sample (PLAP or hCG). Analyte and labeled antibody were preincubated for at least 15 min, allowing equilibrium to be established, before the particles were added.

All particle types were diluted to 310 mg/L in IFMA diluted before addition to the samples. In both assays, 10 μL each of MPa and MPb particles were added simultaneously. The mixtures in the assays were then incubated for 2 h on a horizontal rotational shaker at room temperature. Thereafter, small volumes from each sample were measured by flow cytometry, without prior washing, at a sample injection rate of 5 μL/min for 30 s. To maintain constant incubation times, we added particle suspensions to the assay tubes at 2-min intervals, allowing an analyzing frequency of one sample per 2 min. For comparison, assays without the MPb particles were also performed for both PLAP and hCG.

**FLOW-CYTOMETRIC MEASUREMENTS**

Correlated measurements of fluorescence and light scatter were performed with an Argus flow cytometer (Skatron, Tranby, Norway) equipped with a 75 W mercury-xenon lamp. The filters used provided excitation in the range 510–560 nm and fluorescence detection in the range 590–640 nm. Light-scatter and fluorescence signals from the different particles were measured simultaneously, pulse-processed, and presented in histograms. Distributions of logarithmic fluorescence and light-scatter signals were acquired in 256 discrete channels, covering three log10 orders of signal intensity. The median channel of the logarithmic fluorescence histogram was used as a measurement of the amount of fluorescence bound to each particle. The different particle types were identified by their light-scatter properties, which allowed individual fluorescence determination for each particle type.

**Results**

We have evaluated the binding kinetics of the PLAP assay by analyzing specific binding after various incubation times in samples mixed with different concentrations of the MPa and MPb particles (Fig. 2). If analysis at equilibrium is required, Fig. 2 shows that rapid assay results can be obtained at the expense of sensitivity (signal intensity) by using high particle concentra-

![Fig. 2. Assay kinetics for different concentrations of particles: (A) fluorescence response of PLAP-specific particles (MPa) in a sample containing 73 μg/L PLAP; (B) fluorescence response of the scavenger particles (MPb) in an analyte-free sample.](image-url) Measurements in A and B were performed after 0.5–24 h of incubation. Signals from the NSB particles are shown after 0.5 and 24 h of incubation.
tions. The binding kinetics for the particles used in the PLAP assay is slightly slower than expected from the observations of Frengen et al. [9] in a similar experiment with hCG as the analyte. This may imply a slightly lower affinity of the antibodies used in the PLAP assay than the antibodies used in the hCG assay.

The particle concentration of 310 mg/L for both particle types (MPa and MPb) was measured at 2 h, conditions chosen for the present PLAP ALBA to provide a rapid assay with measurement close to equilibrium. Fig. 3 (left) shows the calibration curves for the specific binding particles MPa and the scavenger particles MPb in the PLAP assay. The median channel numbers of the logarithmic fluorescence measured from the respective particle types were plotted as a function of PLAP concentration. The MPa particles, coated with an antibody against PLAP, gave the expected increase in response to PLAP concentrations of 5–1000 μg/L, but at higher concentrations the hook effect caused a rapid decrease in the MPa response. The MPb particles, which were coated with the antidiotypic antibody with affinity for the label antibody, gave the expected high response at low PLAP concentrations (where the amount of excess label antibody was high), but at PLAP concentrations >10 μg/L the limited amount of uncomplexed labeled antibody caused the MPb response to decrease. As Fig. 3 (left, A) shows,

\[ A \]

**Fig. 3.** Performance of ALBA with PLAP (left) and hCG (right).

(A) Calibration curves for MPa (○) and MPb (▼) after 2 h total incubation; MPa (○) particles in an assay without MPb present; (▼) autofluorescence of MPa and MPb.

(B) Precision profiles for the assayed results of MPa (○) and MPb (▼).
the MPa calibration curve over most of the range is unaffected by the addition of MPb particles. The only influence is advantageous, in that adding the MPb particles reduced the NSB to the MPa particles by 4 fluorescence channel units (i.e., 15% of total NSB) and thus slightly increased the signal-to-noise ratio (data in lower-left corner of panel A).

Precision profiles (CV) for the calibration curves for MPa and MPb (Fig. 3, left, B) were estimated from:

\[
CV = \frac{\sigma_D}{D} \times 100 \text{ (\\%)}
\]

(1)

The standard deviation (\(\sigma_D\)) in the determination of the analyte concentration or dose, \(D\), could for both curves be determined from an estimate of the standard deviation (\(\sigma_R\)) in the measured fluorescence response, \(R\), and the slope of the respective calibration curve, \(k\):

\[
\sigma_D = \left| \frac{\partial D}{\partial R} \right| \sigma_R
\]

(2)

\[
k = \frac{\partial R}{\partial (\log_{10} D)} \Rightarrow \frac{\partial D}{\partial R} = \frac{D}{k \log_{10} e}
\]

(3)

\(\sigma_R = 0.83\) channel units was determined experimentally from intraassay measurements of five parallel determinations of four different PLAP calibrators (0, 30, 1000, and 30 000 \(\mu\)g/L). The assay working range is conventionally determined as the concentrations for which CV values are <10%. The B panels of Fig. 3 show the precision profiles provided by the MPa and MPb calibration curves. The scavenger particle MPb plays a crucial role in providing precision in the middle concentration range, where the specific MPa measurements change from ascending to descending values. The overall precision is optimized by combining the assay results of MPa and MPb, named \(D_a\) and \(D_n\), respectively, in an unbiased, weighted mean value, \(D_w\):

\[
D_w = a \cdot D_a + (1 - a) \cdot D_n \quad a \in [0,1]
\]

(4)

For each pair of \(D_a\) and \(D_n\) measurements, the weighting parameter \(a\) is determined from statistical theory to minimize the variance of \(D_w\), which will be equal to or less than the lower of the variances of \(D_a\) and \(D_n\). With an upper CV limit of 10%, the ALBA provides unambiguous determinations for PLAP dilutions over a working range covering >5 \(\log_{10}\) orders (<5 \(\mu\)g/L to >0.8 g/L).

As an alternative to the PLAP assay, we present another approach to verify the ALBA analysis, this time coating the scavenger particles MPb with the fragment of the analyte that binds the labeled antibody, as illustrated in Fig. 1. Fig. 3 (right, A) shows an hCG assay in which the MPa particles were coated with an antibody against the \(\alpha\)-subunit of hCG and the scavenger MPb particles were coated with the hCG \(\beta\)-subunit. Both particle types were used at 310 mg/L, and the assay tubes were incubated for 2 h before analysis, allowing the binding reaction of the MPa particles to approach equilibrium. Compared with the MPb particles in the PLAP assay (Fig. 2B), the MPb particles in the hCG assay showed much slower binding kinetics, reaching equilibrium only after >24 h of incubation (results not shown). After 2 h of incubation, the MPb particles had bound excess labeled antibody corresponding to >50% of the binding at equilibrium. The MPa response gave the expected calibration curve shape of a one-step sandwich immunoassay, with a pronounced hook effect for hCG concentrations >2 \(\times 10^8\) IU/L. At low analyte concentrations, the large amount of excess label antibody resulted in a high response of the MPb particles. As the amount of excess label antibody decreased for higher analyte concentrations, the MPa response curve descended. As in the PLAP ALBA assay, adding the MPb particles reduced the NSB of the MPa particles, this time by 5 fluorescence channel units (i.e., 24% of total NSB), without otherwise affecting the MPa calibration curve (data in lower-left corner, Fig. 3, right, A). The resulting ALBA calibration curves show the fluorescence response of MPa and MPb with unambiguous determinations for hCG dilutions covering >6 orders of magnitude (8 to >1 \(\times 10^7\) IU/L), according to the CV limit (<10%).

**Discussion**

Particle-based immunoassays read by flow cytometry have previously been shown to allow implementation of highly sensitive homogeneous assays \([14]\). This is possible because the size of the binding region of the flow cytometer is comparable with the dimensions of the microscopic particles. Thus, excess label antibody in solution contributes minimally to the measured specific response from the particle. Still, excess label antibody represents dose-dependent analyte information that has previously been neglected, both in flow-cytometric assays and other conventional immunoassays. Here, we have evaluated a new method for the simultaneous and independent quantification of two \((a)\) label antibody specifically complexed with analyte and \((b)\) excess unbound label antibody. This assay (ALBA), developed in our laboratory, utilizes two distinguishable particle types: one (MPa) that binds the analyte, and one (MPb) that binds excess label antibody. The effect of the latter, scavenger particle (MPb) resembles a washing step by removing uncomplexed label antibody from the solution and thus reduces NSB on MPa. More importantly, we measure the amount of excess label antibody by using the calibration curve for the MPb particles in addition to the ordinary calibration curve for the MPa particles. The MPb curve will show maximum binding for analyte-free samples and a systematically decreasing response as the analyte concentration increases. Hence, plotted together, the resulting set of calibration curves for MPa and MPb provides an unambiguous determination of analyte concentration over an extended working range and eliminates the problems related to the hook effect.

The concentrations of MPa and MPb used in the PLAP assay (Fig. 3, left) were chosen to give a sensitive and rapid assay analyzed close to equilibrium. The lower end of the working range of this assay (<5 \(\mu\)g/L) is comparable with that of other PLAP assays \([17]\). However, combining the calibration curves for both types of particles provides an unambiguous determination of analyte concentration over a working range of >5 \(\log_{10}\) orders (Fig. 3). Because the assay presented has not been systematically optimized with respect to detection limit, further improvements of assay sensitivity can probably be achieved. For instance, use of a lower concentration of MPa will give an
increased signal-to-noise ratio (Fig. 2) and thus lower the detection limit. However, a corresponding increase in the incubation time would be required for analysis at equilibrium.

Preincubation of analyte with label antibody allows this reaction to approach equilibrium before MPb scavenger particles are added. In that case, the MPb particles may be coated with a ligand having high binding affinity for the labeled antibody and thus provide an efficient scavenger function. Noticeable reverse reaction of the analyte-bound label will not be induced during the incubation periods used, as evidenced by the unchanged specific binding to the MPa particle (Fig. 3, both A panels).

In the ALBA of hCG, the scavenger MPb particles are coated with the part of the analyte that binds the label antibody (β-hCG). The β-hCG subunit, rather than the entire analyte, was chosen to prevent possible problems caused by leakage of analyte from the MPb particles. If intact analyte were used, and some of it were lost from the MPb particles, this would add to the amount of analyte in the sample and result in falsely high MPa responses for low analyte concentrations. The binding characteristics of the MPb particles would, however, be practically unaffected by this leakage, because only very small fractions would be assumed to loosen. These alternative assay designs show the versatility of the ALBA, indicating that the scavenger MPb particles may be coated with any ligand having affinity for the antigen-binding site of the label antibody.

Generally, Fig. 3 shows that the reduction of the NSB signal is limited, typically 4 and 5 channel units (i.e., 15–25%), when the scavenger MPb particles are added in rapid assays. From this we conclude there is still potential for further reducing the NSB in these setups, thus further improving the sensitivity of the ALBA analysis. For both assays, preincubation with MPb particles before adding the MPa particles substantially reduced NSB but also the signal-to-noise ratio, thus preventing the use of this procedure (results not shown). Theoretically, if all excess label antibody is removed, the source of NSB will be eliminated, leaving only the autofluorescence of the particles as a limitation in the assay sensitivity. A nonimmunoreactive fraction of label antibody and traces of uncomplexed SA-PE in the assay tubes are probably the main reasons for the limited efficiency of the scavenger MPb particles in the present PLAP and hCG assays. A more pronounced NSB reduction and thereby improved ALBA results can probably be obtained if purified label antibody or directly labeled antibody is used.

The dual-affinity assay of Lindmo et al. [6] and the sequential-binding assay of Frengen et al. [7] are alternative assay designs with qualities similar to the ALBA, both being able to eliminate the hook effect ambiguity and thus increase the assay working range. These assays eliminate the hook effect ambiguity by utilizing two distinguishable particle types coated with antibodies of the same specificity. In both cases, the calibration curves from the two independent particle types show the typical shape of a proportional dose–response sandwich assay curve, but the curves are shifted with respect to each other along the analyte concentration axis. For the dual-affinity assay, this shift was obtained by using antibodies of the same specificity but different affinity on the different particle types. In general, antibodies of identical specificity and different affinity may be hard to come by. For the sequential-binding assay, both particle types were coated with the same antibody, but the particle types were used at different concentrations and added to the sample at different times. This procedure requires at least two processing steps for addition of particles, which complicates the assay design.

In the ALBA setup, all particle types can be added simultaneously, as shown in the hCG and PLAP assays, thus simplifying the implementation of the assay. The ALBA system also provides a more clear-cut identification of the hook effect by the large response differences between high- and low-dose samples in the decreasing MPb calibration curve. Moreover, the ALBA has the potential to remove all excess label antibody and thus minimize the NSB on the MPa particles. The challenge in establishing an ALBA system is to obtain a relevant binding protein for the scavenger particle with the required efficiency. Antidiotypic antibodies against the labeled antibody used in common immunometric assays may not be easily obtained. Alternatively, by coating the MPb particles with the analyte or the label-binding part of the analyte, as in the hCG assay presented, this procedure should theoretically be applicable to a large number of analytes. The ALBA design should generally be considered for assays where an extended working range is required. The NSB-reducing characteristics of this assay should be further investigated by using high-affinity antibodies and direct labeling to yield rapid, highly sensitive assays.

Eliminating the hook effect ambiguity and thus maintaining only one analysis step improves the overall assay speed and makes the ALBA a valuable alternative to other immunometric assays [4, 6, 7]. The ALBA principle is most readily implemented in flow-cytometric immunoassays, where a new measurement parameter can be included by introducing another particle type, distinguishable from the ones already in use by (e.g.) a difference in size. However, the ALBA principle can also be implemented in conventional immunometric assays using microtiter plates by introducing another solid phase that will capture the unused, excess label. Magnetic beads may be a good candidate for such a scavenging solid phase. By subjecting the excess label to measurement rather than washing it off and thereby losing its information, the advantage of the ALBA will be realized.

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