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Protocol

Quantitation of rare memory B cell populations by two independent and complementary approaches

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Abstract

Current methodology for quantitation of memory B cells (MBC) in clinical samples is limited and often does not allow for detection of multiple MBC specificities in a single assay. Here we describe two independent approaches to antigen-specific MBC quantitation. First, a sensitive flow cytometry (FC) assay was developed for simultaneous quantitation of two prototypical B cell antigens, tetanus and diphtheria. Second, an ELISA-based MBC limiting dilution assay (LDA) was developed that provides quantitative analysis of up to 8–12 different MBC frequencies from a single blood sample. Cross-validation studies indicated that MBC numbers measured by FC correlated significantly with the frequencies obtained by LDA ($R^2=0.92$, $p=0.0002$). These two functionally distinct approaches will be useful for accurate quantitation of rare MBC populations specific for either simple antigens (e.g. tetanus and diphtheria) or complex antigens (e.g. vaccinia or other viruses), and is amenable for use in a variety of model systems.

Keywords: B cell memory; Flow cytometric analysis; Limiting dilution analysis; Multiple species

1. Introduction

Enumeration of antigen-specific memory B cells (MBC) in humans is technically challenging, due to their relatively low frequencies in peripheral blood (Leyendeckers et al., 1999). Flow cytometry (FC), even with its high-throughput capabilities, can be hampered by nonspecific binding of some antigens, especially repetitive or complex multi-protein antigens such as intact virus particles (Doucett et al., 2005). In addition, few options are available for examining multiple antigen-specific MBC populations from a single experiment, which would be of great utility when analyzing rare clinical samples.

To overcome these limitations, we have developed two distinct MBC quantitation methods that are applicable to a wide variety of model systems. These assays use FC staining to directly detect antigen-specific MBC by virtue of their affinity/avidity for cognate antigen, or MBC numbers are determined through functional analysis based on antibody production following *in vitro* polyclonal stimulation of purified peripheral B cells under limiting dilution assay (LDA) conditions. Eight parameter FC was used to simultaneously detect tetanus

Abbreviations: DT, diphtheria toxoid; FC, flow cytometry; HAS, human serum albumin; Ig, immunoglobulin; LDA, limiting dilution assay; LPS, lipopolysacharride; MBC, memory B cell; NHP, non-human primate; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen; RM, rhesus macaque; SAC, *Staphylococcus aureus* Cowan strain; TT, tetanus toxoid; UCB, umbilical cord blood.

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toxoid- (TT) and diphtheria toxoid- (DT) specific MBC in human peripheral blood. The ELISA-based LDA was also able to detect TT- and DT-specific MBC, with the potential to quantitate 8 to 12 different antigen-specific MBC populations from a single blood sample. One distinct advantage of developing two independent approaches to MBC quantitation (one based on antigen binding and the other based on antigen-specific antibody secretion) is that these assays can be internally cross-validated in terms of both sensitivity and specificity. Moreover, MBC quantitation by the LDA method is highly amenable to any antigen-specific model system that has a pre-existing ELISA protocol. The ability to enumerate a variety of both simple and complex antigen-specific MBC populations simultaneously will provide a better understanding of the roles that MBC play in the induction, maintenance and anamnestic phases of the humoral immune response following infection or vaccination.

2. Methods

2.1. Animal studies

BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Rhesus macaques (RM) were bred in colonies at the Oregon National Primate Research Center. To induce TT- and DT-specific humoral immune responses, mice were immunized i.p. with 100 μl of Tripedia® (Aventis-Pasteur), followed by two booster immunizations (d21 and d43). Rhesus macaques were immunized with 500 μL Tripedia®, followed by three booster doses administered at one-month intervals. Murine splenocytes were obtained after euthanasia and used immediately or after cryopreservation. Peripheral blood was collected from RM into heparinized tubes, peripheral blood mononuclear cells (PBMC) were purified on Histopaque®-1007 (Sigma-Aldrich) and cryopreserved. The Oregon Health & Science University (OHSU) Institutional Animal Care and Use Committee approved all animal use protocols.

2.2. Human subjects

Umbilical cord blood (UCB) samples were obtained from discarded umbilical cords of full-term deliveries, purified on Histopaque®-1007 (Sigma-Aldrich) and cryopreserved. Adult volunteers provided written informed consent and completed an extensive medical history questionnaire (including TT and DT vaccination dates) before participation in the study. At the indicated time points, peripheral blood was collected into heparinized tubes, PBMC were purified on Histopaque®-1007 (Sigma-Aldrich) and cryopreserved. All human studies were approved by the Institutional Review Board for OHSU.

2.3. Conjugation of MBC detection reagents

Recombinant tetanus toxin C-fragment (Calbiochem) was biotinylated using the EZ-Link™Sulfo-NHS-LC-Biotin kit (Pierce), according to the manufacturers protocol and dialyzed against PBS/0.01% NaN_3 to remove excess biotin. Purified diphtheria toxin (Calbiochem) was conjugated to the succinimidyl ester of either Pacific Blue™ or Alexa Fluor® 700 (Molecular Probes). For both conjugates, 250 μg of protein was incubated with dye at a molar ratio of 3.5/1 moles of dye per mole of protein for 1 h at room temperature and purified over P-30 fine resin columns (Bio-Rad). HSA (Sigma-Aldrich) was biotinylated or conjugated to Alexa Fluor® 700 as described above.

2.4. MBC analysis by FC

Cryopreserved mouse splenocytes were stained with α-CD19 (Caltag, clone 6D5) and α-IgD (Southern Biotech, clone 11-26). Human PBMC were stained with α-CD20 (Caltag, clone HI47) and α-IgD (BD Biosciences, clone IA6-2). RM PBMC were stained with α-CD20 (Caltag, clone HI47) and α-IgD (Southern Biotech, Goat pAb, δ heavy chain specific). To enumerate TT- and DT-specific MBC, cells ($10–30\times10^6$) were stained with 0.3 μg of rTT.C-FITC (List Biological Laboratories), 0.1 μg of rTT.C-biotin, 0.16 μg of DT-Pacific Blue™, and 0.33 μg of DT-Alexa Fluor® 700. Control stains were carried out with 0.1 μg of HSA-biotin or 0.16 μg of HSA-Alexa Fluor® 700. Cells were stained in 50 μl volumes for 1 h at 4 °C, washed, and incubated with streptavidin-APC (diluted 1/500, Molecular Probes) for 30 min at 4 °C. Cells were washed again and fixed with 2% formaldehyde in PBS. Events were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.) with live cell gating based on FSC × SSC characteristics.

2.5. Human MBC limiting dilution analysis

Initial optimization experiments with whole PBMC indicated that antibody responses were inhibited at high cell concentrations and detection of low frequency antigen-specific MBC ($<20/10^6$ PBMC) required B cell purification prior to *in vitro* culture. B cells were isolated from total human PBMC through positive