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Identification of antigen-specific human monoclonal antibodies using high-throughput sequencing of the antibody repertoire

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ABSTRACT

High-throughput sequencing of the antibody repertoire provides a large number of antibody variable region sequences that can be used to generate human monoclonal antibodies. However, current screening methods for identifying antigen-specific antibodies are inefficient. In the present study, we developed an antibody clone screening strategy based on clone dynamics and relative frequency, and used it to identify antigen-specific human monoclonal antibodies. Enzyme-linked immunosorbent assay showed that at least 52% of putative positive immunoglobulin heavy chains composed antigen-specific antibodies. Combining information on dynamics and relative frequency improved identification of positive clones and elimination of negative clones. and increase the credibility of putative positive clones. Therefore the screening strategy could simplify the subsequent experimental screening and may facilitate the generation of antigen-specific antibodies.

1. Introduction

High-throughput sequencing of the antibody repertoire has been widely used in basic and applied immunology research, in particular for the generation of antibodies [1,2]. Although this method rapidly provides a large number of antibody variable region sequences, further improvements are needed to increase the screening efficiency. One approach combines proteomics with high-throughput sequencing to identify antigen-specific antibodies directly from the serum of immunized animals [3]. This work was also successfully applied to the identification and cloning of antigen-specific human monoclonal antibodies [4], but a simpler and less expensive method is desirable.

Another approach to antigen-specific antibody screening is the sorting of antigen-specific antibody secreting cells (ASCs) and memory B cells (mBCs) [5]. However, antigen-specific mBCs usually account for only a small fraction of circulating or total class-switched mBCs [6,7], making screening difficult. Similarly, differentiated ASCs express low levels of B cell receptors (BCR), which results in poor antigen-binding capacity and makes them difficult to detect [5,8]. Furthermore, mBCs or ASCs may express cell surface receptors of some antigens, such as protective antigen (PA) of in *Bacillus Anthracis* (unpublished data), which limits antigen-specific cell sorting applications. The cellular affinity matrix method reported by Shimizu and Azuma to detect and isolate anti-hapten ASCs [8] could prove useful for obtaining antigen-specific antibodies, but it remains complicated and challenging for isolation of complex antigens.

Some researchers have attempted to obtain antibodies based on the relative frequency of sequences [9,10], and a high proportion of the most abundant clones showed antigen-binding capacity. However, the experimental mice used in these studies have a simpler immune background than humans. Thus it would be more inefficient and difficult to screen antigen-specific human antibodies than antigen-specific mouse antibodies using this strategy. Furthermore, bone marrow and lymph node samples used in these mouse studies are rarely available from humans.

One pivotal study characterized the rapid changes in antibody dynamics over time in response to an immune challenge in multiple individuals [11]. Interestingly, some clones were present throughout the entire observation period, while others appeared in

Abbreviations: ASCs, antibody secreting cells; BCsR, B cell receptors; CDRs, complementarity determining regions; ELISA, enzyme-linked immunosorbent assay; IgH, immunoglobulin heavy chain; IgK, immunoglobulin kappa chain; mBsC, memory B cells; PA, protective antigen; PBMCs, peripheral blood mononuclear cells; PBST, PBS containing 0.1% Tween 20.

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response to vaccination [11]. Paying attention to both the dynamics and relative frequency of clones may therefore improve the efficiency of screening antigen-specific human antibodies.

In the present study, we vaccinated a volunteer with a recombinant PA anthrax vaccine, and selected clones based on characterization of dynamics and relative frequency. Enzyme-linked immunosorbent assay (ELISA) results showed that positive immunoglobulin heavy chain (IgH) clones accounted for 52% of total predicted positive clones. Consideration of clone dynamics reduced false positives from the immune background and increased the efficiency of antigen-specific human antibody screening. This approach could be applied to the generation of antigen-specific antibodies.

2. Materials and Methods

2.1. High-throughput sequencing of the antibody repertoire

After informed consent was obtained, a healthy male volunteer was vaccinated with the recombinant PA anthrax vaccine (an investigational anthrax vaccine developed by the Beijing Institute of Biotechnology, Batch number 201402YA01) and a second booster vaccination was administered 28 days later. Blood samples were collected and peripheral blood mononuclear cells (PBMCs) were obtained on days 0, 7, 28, and 35 using a density gradient method (Dakewe Biotech, Beijing, China). Total RNA from PBMCs was isolated with the RNeasy Plus Mini Kit (QiaGen, Hilden, Germany). Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA) and oligo(dT)20 primer. The resulting cDNA was amplified with TransStart Taq DNA Polymerase (TransGen Biotech, Beijing, China) and a gene specific primer set [12,13]. PCR products were separated by electrophoresis and bands approximately 500 bp in size were purified. PCR products were uniquely coded, ligated with sequencing adaptors, and sequenced using the Illumina MiSeq platform.

2.2. Sequencing data processing and analysis of clone dynamics

To annotate the recovered sequences, paired-end Miseq sequencing results were filtered and merged before analysis using the international ImMunoGeneTics (IMGT) information system database HighV-QUEST (IMGT/V-QUEST program version 3.3.4) [14]. Each IgH and immunoglobulin kappa chain (IgK) complementarity determining region (CDR) 3 was counted at each time point to calculate the CDR3 frequency and observe the CDR3 dynamics. The results were plotted using R scripts which are available upon request.

2.3. Clone screening strategy

For IgH CDR3 sequences, the 100 most frequently observed clones were selected for further investigation. If the frequency of a clone on day 0 was not 0, the clone was assigned to group HZ1. Clones with a frequency on day 28 that was higher than on day 35 were assigned to group HZ2. Clones with a frequency on day 28 that was higher than on day 7 were assigned to group HZ3 (true) and group HZ4 (false). A different number of clones from each group were selected (see results and discussion). For each IgH CDR3 clone, the sequence of the complete variable region was determined by grouping all variable region sequences containing the CDR3 based on their CDR1 and CDR2 sequences. A single clone from the largest group was selected and its nucleotide sequence was chosen as the variable region sequence to be analysed.

For IgK CDR3 sequences, the 100 most frequently observed clones were selected for further investigation. Clones with a frequency on day 0 that was not 0 were assigned to group KZ1, and the others were assigned to group KZ2. A different number of clones in either group were selected (see results and discussion). The sequence of the complete variable region of each IgK CDR3 clone was determined as described above.

2.4. Construction of expression vectors

Vectors derived from the pcDNA3.4 plasmid were used for expression of an antibody kappa chain and an IgG1 heavy chain. Briefly, four short sequences (S1, CTATGGGTATCTGGTACCTGTGGG in the leader region of the first vector; S2, CTGTGGCTGCACCATCTGTCTTC in the constant region of the first vector; S3, CTAATTTTAAAAGGTGTCCAGTGT in the leader region of the second vector; S4, CCAAGGGCCCATCGGTCTTCCCC in the constant region of the second vector) were selected as homologous arms, and point mutations were performed to introduce an NheI restriction enzyme site just downstream of S1 and S3, and a BamHI site just upstream of S2 and S4. Variable region sequences from selected clones (containing S1 and S2 for the kappa chain and S3 and S4 for the heavy chain) were synthesized (Genewiz, Suzhou, China). After the modified vectors were linearized with NheI and BamHI (New England Biolabs, Ipswich, MA), the synthesized genes were ligated into the corresponding vector using a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech).

2.5. Expression of recombinant antibodies

The HEK293T human embryonic kidney cell line (ATCC CRL-11268) was seeded into 96-well flat-bottomed cell culture plates (Coring Incorporated, Kennebunk, ME) at a density of 3×10^4 cells/well and incubated at 37 °C overnight. Cells were transfected with 200 ng of the IgH and IgK plasmids using TurboFect Transfection Reagent (Thermo Scientific, Rockford, IL). After 60 h, the culture supernatant was collected.

2.6. ELISA

ELISA was performed to determine the antibody response to the rPA vaccine and to screen antibodies expressed for PA-specific binding activity.

(1) ELISA to determine antibody response to the rPA vaccine.

EIA/RIA stripwell plates (Coring Incorporated) were coated with 200 ng of rPA (prepared as described previously [15]) in 100 μL coating buffer (0.05 M carbonate buffer solution, pH 9.6) overnight and blocked with 200 μL blocking buffer (PBS containing 0.1% Tween 20 and 2% BSA). Plates were washed with PBS containing 0.1% Tween 20 (PBST), and serial dilutions (a 2-fold series of 1:10 dilutions unless otherwise noted) of plasma samples were added to each well. Plates were incubated for 1 h at 37 °C, washed with PBST, and HRP-conjugated anti-human IgG antibody (Sigma–Aldrich, St. Louis, MO) was added. Plates were incubated for an additional 1 h at 37 °C, washed again in PBST, and colour was generated using 3,3′,5,5′-tetramethylbenzidine substrate (Solarbio, Beijing, China). The reaction was stopped with 2 M H_2SO_4, and the optical density was measured at 450 nm. A standard curve was plotted against a sample containing 48.0 μg/mL anti-PA IgG (a 2-fold dilution series from 1:40 to 1:1280), and the concentration of the PA-specific IgG in the experimental sample was determined.

(2) ELISA to screen PA-specific antibodies.

EIA/RIA stripwell plates were coated with 200 ng recombinant