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Single B cell antibody technologies

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Monoclonal antibodies (mAbs) are arguably the most significant class of biologics for use as pharmaceuticals and diagnostics. Many technological concepts exist for the generation and identification of therapeutically relevant mAbs, including the isolation and cloning of immunoglobulin (Ig) encoding genes from single B-lineage cells. This review summarizes various single B cell approaches and describes their use for the discovery of mAbs with potential therapeutic values or in basic research.

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Introduction

Monoclonal antibodies (mAbs) are essential tools for many diagnostic applications and research investigations. In addition, mAbs have become ideal therapeutic reagents for many diseases and are well established in the treatment of cancer, autoimmune disorders and infectious diseases [1]. Many technologies exist for the generation of mAbs for both research and therapeutic purposes. Generally, for research purposes, murine mAbs are obtained by hybridoma technology that is based on the fusion of B cells, for example derived from immunized mice with myeloma cells [2]. In this way, mAbs against almost any antigen are created, but the direct therapeutic use of murine mAbs in humans is limited by both the high incidence of harmful immune responses against the murine, foreign protein and the murine antibodies lack of sufficient effector function [3,4]. To alleviate these limitations, murine mAbs have been reengineered by chimerization and humanization technologies in which the murine constant and framework regions are replaced by human sequences [5–8].

Alternatively, human mAbs can be recovered from hybridoma B cell lines derived from immunoglobulin (Ig) transgenic mice with complete or partial human antibody repertoires [9–12]. One drawback is that the Ig transgenic mouse systems cannot precisely imitate a human immune response because of the effects of the murine genetic background on antigen processing and B cell regulation. As a result, the recovered antibodies might not display the precise specificities of naturally occurring antibodies in humans. Despite this drawback, the majority of the approved fully human antibodies today have been derived from Ig transgenic mouse systems.

To overcome the issues of murine systems, strategies for engineering mAbs created by an intact human immune system have been designed, which include the human hybridoma technology.
[13–15] and the immortalization of human B cells by transformation with Epstein-Barr virus (EBV) [16,17]. One major advantage of these methods is that they allow the isolation of native human mAbs with the preservation of the natural VH and VL pairing. These methods, however, are generally unsuitable for a comprehensive screening of large antibody repertoires, because of their highly selective and inefficient fusion and transformation events. In spite of this, improvements of these methods have yielded neutralizing mAbs, for example, against the H1N1 [18] and the H5N1 [19] influenza virus or the SARS corona virus [20].

Additional ways for creating and selecting mAbs include combinatorial display technologies, like phage, yeast and mammalian cell display [21]. Phage display has been successfully employed in the screening of large antibody repertoires and the isolation of mAbs against virtually any antigen of interest [22]. The combinatorial display technologies also allow for in vitro affinity maturation of selected antibodies [23,24]. One drawback, however, is that they usually rely on random combination and thus most likely unnatural VH and VL antibody pairings.

As it is thought to be important in antibody development to maintain the original VH and VL pairing as exists in human B cells, an efficient strategy has been designed which is based on the direct amplification of VH and VL region encoding genes from single human B cells and their subsequent expression in cell culture systems [25–32]. To facilitate rapid screening and detection of single cells secreting antibodies with the desired reactivity profiles from large populations of primary B cells cell-based microarray and microengraving techniques can be applied [33–37]. The single B cell antibody approaches harbor the potential to isolate functional mAbs reactive against conformational determinants that are present predominantly in vivo and difficult to emulate in vitro. The strategies straightforwardness, the requirement of relatively few cell numbers and the high efficiency in obtaining specific mAbs in a rapid way are balanced by the downsides, which are the need of adequate human donors and the limitation to certain target molecules. The following paragraphs describe the basic concept of single B cell antibody technologies and their use in basic research and in the selection of antigen-specific mAbs.

Concept of single B cell antibody technologies

Identification and isolation of single B cells

Depending on the research application, single B cell isolation can be performed either in a random way or in an antigen-selective manner from peripheral blood or from lymphoid tissues, for example the bone marrow. For random B cell isolation, procedures such as, cell picking by micromanipulation [38], laser capture microdissection [39] and fluorescence-activated cell sorting (FACS) [30,32,40] have been described. Alternatively, selection of antigen-specific B cells has been demonstrated using antigen-coated magnetic beads [28], fluorochrome-labeled antigens via multi-parameter FACS [41–43], the hemolytic plaque assay [25] and a fluorescent foci method [44]. Furthermore, cell-based microarray chip systems [33,34] have been established for high-throughput screening of mAbs secreted by single antibody producing cells.

The major advantage of FACS technology in this application is that cells to be sorted can be clearly distinguished in terms of their stage of development and differentiation based upon the expression patterns of specific cell surface markers. In general, B cells at any stage can be sorted, but class-switched memory B cells and antibody-secreting cells (ASCs, i.e. plasmablasts and plasma cells) are of special interest to obtain relevant mAbs as they bear somatically mutated B cell antigen receptors (BCRs) with high affinities. For example, IgG+ memory B cells reactive to recombinant trimeric gp140 antigen have been successfully selected in patients with HIV by using fluorescently labeled gp140 [43,45]. By contrast, ASCs specific for a vaccine can be sorted without any antigenic labeling post immunization. For efficient recovery of specific mAbs, the donor’s immune response must be assessed in this method, for example, by determining the frequency of ASCs in peripheral blood using an enzyme-linked immunospot technique (ELISPOT) before single cell isolation [30,46].

Additionally, high-throughput methods have been generated that lead to the efficient identification of B cells having the desired specificity before Ig gene cloning. One specific method is a soft lithographic method for microengraving [34,35]. Per screen up to one hundred thousand polyclonal B cells stimulated with a mitogen to develop into ASCs are deposited individually into an array of microscale wells on a chip. The array is then used to print a corresponding protein array, whereby each element contains the antibodies secreted by one single B cell. The antibodies on the microarray are screened with fluorescently labeled antigen(s) of interest, and mapped to the corresponding cells, which are then manually retrieved from their wells by micromanipulation and subsequently analyzed by clonal expansion or single cell reverse transcription-polymerase chain reaction (RT-PCR). A similar approach is the ‘immunospot array assay on a chip’ (ISAAC) method established by Muraguchi’s group [33]. As for the microengraving technology, single ASCs are placed on a microwell chip, but the chip’s surface is coated with anti-Ig antibodies, so that the antibodies secreted by one individual ASC are captured on the surface around the well. The binding of biotinylated antigen to specific antibodies allows the detection with fluorescence-conjugates of streptavidin. Microengraving and ISAAC offer compelling advantages, such as the early and rapid identification of cells secreting antibodies with high affinities specific to an antigen of interest from a polyclonal mixture. Furthermore, these techniques simplify the screening of multiple antigens to allow the selection of different clones with distinct specificities.

Single cell Ig gene transcript amplification, sequencing and cloning

The construction of complementary DNA (cDNA) from single B cells provides an unbiased approach to the simultaneous analysis of expressed IgH and IgL chain genes [47,48]. Usually, single cell cDNA synthesis is performed in the original device that was used for cell deposition and cell lysis (e.g. the 96-well plate), which assures a convenient handling of large numbers of samples and minimizes the risk for cross-contamination. Notably, distinct B cell types show different amounts of specific Ig gene mRNA transcripts. For example, compared to memory B cells, ASCs contain relatively large amounts of Ig gene transcripts, thereby facilitating specific Ig gene amplification. Usually, full-length Ig gene transcripts are amplified by nested or semi-nested RT-PCR, whereby the RT might be performed as one-step reaction with the first round of PCR. Typically, forward primer mixes complementary to the corresponding IgH and IgL V gene leader sequences
and a single reverse primer specific to the constant region sequence are used [32,49–51]. If necessary, for example, when isotype independent cell sorting is performed, mixed reverse primers can be used for the amplification of IgH chains with different constant regions. In the second round, nested primer or primer mixes are used to increase sensitivity and specificity. Furthermore, restriction sites for subsequent cloning steps can be incorporated into the amplicons during this step. Alternatively, linear expression cassettes can be assembled that are directly transfected into mammalian cells for in vitro expression of the amplified Ig genes [52].

Strategies to link the rearranged IgH and IgL chain genes during amplification have also been explored [29,53]. For example, Meijer et al. developed a multiplex single cell RT-PCR approach that combines the amplified IgH and IgL chain products during the PCR through an overlap extension step. The joined DNA fragments are subsequently cloned into an appropriate expression vector for mAb production.

Although single cell RT-PCR methods are straightforward, not every protocol amplifies all functional IgH and IgL V genes because of limitations in the designed set of forward primers used. A method that allows the amplification of unknown 5’ ends of mRNAs is the ‘5’ rapid amplification of cDNA ends’ (5’-RACE) technology. Although standard 5’ RACE techniques are unsuited for synthesizing cDNAs from single cells because of the usually small quantity of mRNA present, an improved protocol has demonstrated its suitability for the isolation of Ig genes from single B cells with promising efficiencies [54].

Regardless of the Ig gene amplification strategy used, the single cell Ig gene transcript information that encodes the specificity of the antibody is subsequently rescued by sequencing. The rearranged V, D and J gene segments can easily be identified and analyzed for the presence of mutations, insertions and deletions [55,56] using various databases, such as the IgBLAST search engine at the NCBI website (http://www.ncbi.nlm.nih.gov/igblast/).

**Reactivity screening of antibodies**

To determine the reactivity profile and biophysical characteristics of antibodies or fragments thereof, the proteins have to be expressed, purified and tested in various assays. Conveniently, in the cell-based microarray systems this reactivity screening can be performed before Ig gene cloning. To analyze the corresponding antibodies in more detail, however, molecular Ig gene cloning steps are indispensable, as large-scale amounts of protein are needed. The most common choices for expression systems are bacterial systems (e.g. *Escherichia coli*) or transient and/or stable mammalian cell systems (e.g. HEK 293, CHO cells). In *E. coli*, the cloned Ig genes are typically expressed as antigen-binding fragments (Fabs), whereas in mammalian cells expression can be in complete IgG format.

**Single B cell antibody repertoire studies**

Classic mouse and human antibody repertoire analyses on a cell-by-cell basis have significantly increased our knowledge about generic B cell immunology concepts. Initially, Ig genes from single B cells were studied on the genomic level by single cell PCR to elucidate the molecular basis of Ig gene rearrangement and allelic exclusion events [38,57–60]. As a next step, strategies were developed for the synthesis of cDNA from single B cells [48] to facilitate the unbiased description of mouse [47,61] and human [62] antibody repertoires. Substantial advances in understanding the expressed human antibody repertoire have then been made by the Nussenzweig group, which pioneered the investigation of defined B cell specificities during B cell development and differentiation [63]. The analysis of recombinant mAbs cloned from individual B cells of healthy individuals revealed that the majority of newly generated B cells in the bone marrow are self-reactive and that two checkpoints – one in the bone marrow and another in the periphery – select against self-reactivity during B cell development [40]. By characterizing alterations in the B cell repertoire of patients with deficiencies in innate immune pathways, the Meffre group suggested that such pathways are required for the removal of self-reactive immature B cells [64]. Based on the analysis of B cells from autoimmune patients it was proposed that a failure to establish tolerance at these checkpoints is associated with the development of Systemic Lupus Erythematosus (SLE) [65,66] and Rheumatoid Arthritis [67]. Further studies described a third checkpoint that selects against self-reactivity during IgM+ memory B cell differentiation [68]. Surprisingly though later in development, IgG+ memory B cells frequently express self-reactive antibodies in healthy donors [69,70]. In addition to these approaches which elucidate basic B cell biology, systems have evolved to isolate valuable mAbs from immune donors for potential therapeutic use or other applications, such as epitope discovery or vaccine design. Because of ethical concerns, humans cannot be exposed to any antigen at will. Therefore, it is clear that adequate donors have to be available from whom ASCs or memory B cells with antibodies reactive to an antigen of interest can be recovered. Particularly, vaccinated human subjects that can be followed up 6–14 days after immunization are ideal candidates from which to obtain such antibodies. As the immune system usually mounts a broad humoral response against an immunizing agent, mAbs reactive to multiple epitopes of the immunogen may be available. This was elegantly demonstrated by the Wilson lab, which engineered over 50 human antibodies specific to an influenza virus vaccine from ASCs isolated from vaccinated donors at day 7 after immunization [46]. Interestingly, as many as 80% of ASCs purified at this timepoint were influenza specific. The recovery of ASCs from peripheral blood at the peak of the immune response is essential, as these cells rapidly decline from the bloodstream as they migrate to the bone marrow to become long-lived plasma cells, which maintain humoral memory for a lifetime [71].

Alternatively, memory B cells can be isolated from the peripheral blood of naturally infected or vaccinated humans even months to years after antigen exposure. Although the exact correlation between antigen-specific memory B cell frequency and serum antibody titers has still to be clarified in humans, the determination of antigen-specific serum antibody titers is helpful in identifying appropriate donors. For example, Scheid et al. characterized the B cell memory response against HIV in six infected patients with broadly HIV neutralizing serum antibodies. In this study, 502 mAbs were isolated from single HIV-envelope binding memory B cells, 433 of them showing specific binding to recombinant gp140 antigen. Although none of the isolated mAbs was found to be broadly neutralizing across various strains of HIV, the combination of individual antibodies revealed a broad neutraliz-
ing effect [45]. A similar approach has produced neutralizing antibodies reactive to over 90% of circulating HIV-1 isolates [72]. Overall, these findings provide important insights into the immune response to HIV and are indispensable for rationale vaccine design strategies. Furthermore, single cell Ig gene cloning has revealed the kinetic and diversity of the antibody response against tetanus toxoid [73] and the Ig gene repertoire of anti-RhesusD-specific B cells [74]. In addition, recently, by using cell-based microarrays, mAbs against hepatitis B surface antigen have been prepared and characterized [75]. Overall, these studies demonstrate the versatility of single B cell antibody approaches in the production of valuable antigen-specific antibodies.

Summary and outlook

Over the last decade, single B cell antibody technologies have become an attractive approach to sample naïve and antigen-experienced antibody repertoires generated in vivo. The method has proved to be very useful in evaluating basic B cell biology concepts in health and autoimmunity. Furthermore, valuable mAbs, have been isolated from vaccinated [46], naturally immunized donors [45] and patients with autoimmune disease [76]. In general, mAbs recovered by single B cell antibody technologies may be useful reagents for diagnostics, pharmacokinetic studies and clinical development. Major applications include the prevention of infectious diseases by passive immunization [77] and therapies for disorders currently medicated with serum IgG preparations [78]. A deeper understanding of neutralizing mAbs isolated from immune donors will also be helpful for rationale structure-based vaccine designs. Promising efforts in this reverse vaccinology approach are currently being made in HIV research, and should be applicable in other infectious diseases. In addition, future advancements on microfluidic platforms, including PCR cycling combined with next generation sequencing methods are likely to exceed the current systems and allow for high-throughput Ig gene sequence analyses of individual antibody repertoires. By increasing the availability of such repertoires, single B cell antibody technologies are likely to lead in the development of novel mAb therapeutics.

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