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The Use of Somatic Hypermutation for the Affinity Maturation of Therapeutic Antibodies

Peter M. Bowers, **William J. Boyle**, and **Robert Damoiseaux**

Abstract

The engineering of antibodies and antibody fragments for affinity maturation, stability, and other biophysical characteristics is a common aspect of therapeutic development. Maturation of antibodies in B cells during the adaptive immune response is the result of a process called somatic hypermutation (SHM), in which the activation-induced cytidine deaminase (AID) acts to introduce mutations into immunoglobulin (Ig) genes. Iterative selection and clonal expansion of B cells containing affinity-enhancing mutations drive an increase in the overall affinity of antibodies. Here we describe the use of SHM coupled with mammalian cell surface display for the maturation of antibodies in vitro and the complementarity of these methods with the mining of immune lineages using next-generation sequencing (NGS).

Keywords

Somatic hypermutation (SHM); Fluorescence-activated cell sorting (FACS); Antibody; Affinity maturation; Heavy chain (HC); Light chain (LC); Complementarity-determining region (CDR)

1 Introduction

Antibody therapeutics have become the leading source of new medicines for the treatment of diseases in a wide cross section of therapeutic areas [1]. There are multiple antibody and antibody- like molecules that have been extensively engineered for clinical use [2, 3]. A common trend observed for approved therapeutics is the use of humanized and/or fully human sequences, regardless of the antibody format, including full-length immunoglobulin G (IgG), monoor bispecific scaffolds, antigen-binding fragment (Fab), and single-chain (scFv) antibodies.

A majority of approved therapeutics, those in clinical and pre-clinical development, are derived from monoclonal antibodies generated in rodents that have been immunized with target antigens. These antibodies have undergone B-cell selection for target binding and stability [4, 5] and later for eliciting an in vitro and in vivo biological effect capable of altering a disease pathway. The direct use of the resulting antibodies is not possible due to immune reactions and subsequent formation of antidrug antibodies (ADAs) upon administration into patients. The process of humanization is employed to transform the rodent antibody scaffold into a human-like immunoglobulin that does not elicit these

[4]Notes

responses while retaining biological activity. First, the rodent immunoglobulin is transformed into a chimeric Ig in which the constant regions are replaced with the orthologous human IgG1 to possess appropriate effector functions (associated with each isotype) and to retain the desired biophysical and pharmacokinetic properties. The second step involves the replacement of the variable gene framework regions with the homologous regions from germline human V-gene orthologs while retaining the rodent-derived CDR sequences, historically termed CDR grafting [6–8].

Historically, the humanization process leads to the generation of lead antibody candidate molecules for characterization and selection of a clinical candidate [9, 10]. In addition, humanized immunoglobulins often do not retain the same characteristics of the original rodent monoclonal antibody with respect to affinity, biological activity, thermal stability, and expression at high levels as a recombinant protein. For example, there is often a significant reduction in binding affinity that makes the first-generation humanized molecule not suitable for clinical use, and the biological activity of the resulting molecule may not be recapitulated. These deficiencies may require significant re-engineering of the humanized antibody to regain traits desired for clinical use and may involve back mutations to the rodent antibody sequences which can compromise the humanization process and lead to immunogenicity issues. Likewise, antibodies derived directly from phage libraries and transgenic mice with humanized antibody repertoires may require further affinity maturation and optimization.

Recently, a process was described in which a single humanized lead antibody was used to generate a host of related molecules by exposing the heavy and light gene variable sequences to regulated SHM [11]. Selection for improved antigen binding of evolved antibodies by FACS binding to fluorescently tagged antigen, followed by NGS analysis, provided an array of sequences that could be computationally assessed to more rapidly obtain optimized candidates for clinical development [12]. In this review we provide an outline for this process which can add significantly to the humanization process.

2 Materials

1. Fugene HD (Roche Applied Science).
2. Dulbecco’s Modified Eagle’s Medium (DMEM).
3. HEK293 c18 (ATCC).
4. Puromycin.
5. Fetal bovine serum.
6. Bovine serum albumin.
7. Phosphate-buffered saline.
8. Opti-Mem (Invitrogen).
9. Accutase (Stem Cell Technologies).

10. FACS buffer: phosphate-buffered saline (PBS), 10% (w/v) bovine serum albumin (BSA), pH 7.4.
11. TrypLE™ Express (Invitrogen Life Science Technologies).
12. Streptavidin Dylight 649 (Vector Labs).
13. PE mouse anti-human IgG Fc Antibody (BioLegend).
14. Alexa Fluor™ 647 Protein Labeling Kit (Thermo Fisher).
15. Recombinant protein (example human IL-13) (PeproTech).
16. Propidium iodide (Sigma-Aldrich).
17. Activation-induced cytidine deaminase (Accession # NP_001003380.1) (see Note 1).

3 Methods

3.1 Cell Line Selection for AID- Mediated In Vitro Affinity Maturation

A cell line for in vitro SHM should be selected that enables cell surface presentation of antibody or antibody libraries, expression of AID, cell sorting by flow cytometry, rapid growth, and easy manipulation for cell and molecular biology methods. AID-mediated mutation has been demonstrated in prokaryotic cells [13, 14] but lacks secondary DNA replication mechanisms such as base excision repair (BER), double strand brakes, and mismatch repair (MMR) that yield non-GC-based mutations in B cells [15], and presentation of full-length antibody on prokaryotic cells is challenging [16]. Yeast display is another potential platform for the display and isolation of antibody fragments via flow cytometry [17] but suffers from the same limitations as prokaryotic display. In the B cells undergoing SHM, housekeeping mechanisms are diverted from standard roles in monitoring DNA integrity to increase diversity within the Ig locus, particularly V-gene CDRS, in response to AID-generated lesions, generating a balanced spectrum of point mutations at adenine, thymine, guanine, and cytosine, as well as insertions and deletions (INDELS) [18]. B-cell-derived cell lines (e.g., Ramos and Raji) possessing native AID activity have been used to affinity-matured antibodies but are difficult to transfect with diverse library repertoire. In non-B-cell eukaryotic cell lines, BER and MMR are not optimized for generation of Ig diversity, leading to mutations skewed toward bases guanidine and cytidine, but mutations at bases adenine and thymine are observed, as are INDELS.

3.2 Transfection of 293-c18 Cells in T-75 Flask Using Fugene HD Transfection Reagent

3.2.1 Generation of a Kill Curve—For selectable markers, a kill curve is necessary to predetermine the appropriate amount of drug necessary to cause complete cell death when using a new cell type/line. Below is a minimal protocol for generating a kill curve.

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1. Activation-induced cytidine deaminase protein sequence:
MDSLLMKQRKFLYHFKNVRWAKGRHETYLCYVVKRRDSATSFSLDFGHLRNKSGCHVELLFLRYISDWDLDPGRCYRVTW
FTSWSPCYDCARHVADFLRGYPNLSLRIFAARLYFCEDRKAEPEGLRRLHRAGVQIAIMTFKDYFYCWNTFVENREKTFKAW
EGLHENSVRLSRQLRRILLPLYEVDDLRDAFRTL.

Identifying the appropriate concentration of puromycin (or equivalent antibiotic) for a new cell line:

1. Plate 1E + 04 cells per well into a 384-well plate.
2. ncubate overnight at 37 ○C.
3. The following day add 500–10,000 ng/mL puromycin to selected wells leaving control wells untreated.
4. Replace media containing puromycin every 3 days over 6–10 days.
5. Remove media, wash with PBS and apply Accutase or TrypLE to release cells.
6. Resuspend in FACS buffer.
7. Run FACS for number of live cells to determine minimum concentration which causes complete cell death. Compare to untreated control.

3.2.2 Stable Transfection of HEK293-c18 Cells with Antibody HC/LCs—Cell surface display and maturation of IgG on HEK293 or CHO cells have been demonstrated through stable dual expression of a heavy and light chain constructs together with stable or transient expression of AID. The human IgG1 heavy chain is fused at its carboxyl terminus to a short juxta-membrane region, the trans-membrane domain and intracellular domain from the mouse H-2Kk protein (Fig. 1).

1. Prepare HEK293-c18 cells at a cell density 1E + 06 cells/mL in DMEM containing 10% FBS.
2. Add 3 mL of cells per T-75 flask.
3. Dilute the cells with 7 mL of DMEM containing 10% FBS.
4. Incubate overnight at 37 ○C.

Next day transfect with the following:

5. Dilute Fugene HD (room temperature) by pipetting 20 μL of the transfection reagent into 500 μl OptiMEM (room temper- ature) without allowing contact with the walls of the plastic tube. Vortex for 1 s or flick the tube to mix and incubate for 5 min at room temperature.
6. Add 4 μg plasmid DNA encoding dual HC/LC expression (example sequences as seen in Fig. 1) to the diluted Fugene HD, tap the tube, or vortex for 1 s to mix the contents.
7. Incubate for 25 min at room temperature.
8. Add the transfection reagent-DNA complex to the cells in a dropwise manner.
9. After 48 h apply Accutase or TrypLE to cells and incubate for 3 min, loosen cells, and inactivate trypsin with 9 mL DMEM containing 10% FBS with puromycin.

10. Plate 1E + 06 cells in T-225 in total volume of 50 mL DMEM containing 10% FBS with puromycin and select with antibiotics.

11. Flow cytometry can be used to isolate HEK293-C18 cells possessing high, moderate, or low levels of surface IgG expression, depending on the affinity of the antibody in question.

3.3 Transient AID Transfection

While cell lines can be stably transfected with AID, the cytidine deaminase activity may act on other highly expressed genes in the genome and create targeted mutation. For example, AID activity is known to be upregulated in various cancers [20], presumably as an adaptive, evolutionary mechanism altering the function of many genes. As such, the long-term stability and maintenance of cells expressing high levels of AID may be problematic, and the transient expression of AID in antibody-producing cell lines may be preferable. Foreither stable or transfected AID approaches, modifications of AID can be explored that promote increased AID activity and durability. Mutations that disable the nuclear sequence located at the C-terminus of the protein and the introduction of a nuclear localization sequence at the N-terminus can be used to promote increased AID activity in the nucleus. Likewise, the degeneracy of the genetic code can be used to remove known AID motifs [12], thereby preventing auto- inactivation of AID enzymatic activity. Use of transient AID transfect to drive evolution circumvents this potential problem (Fig. 2).

1. Passage antibody-expressing cells, exchanging media and anti- biotic every 3 days.

2. 6–7 days prior to flow cytometry sorting, perform a transient transfection of AID using ~4E + 06 cells using conditions similar to those outlined above, anticipating 3E + 07 cells for cell staining and flow sorting.

3.4 Flow Cytometry Sorting of Cells Expressing Antibody Variants with Improved Binding to Antigen

After stabilization of the antibody-expressing cell line, FACS sorting for desired expression of surface IgG and antigen binding will be used to perform repeated rounds of affinity maturation as follows. Each round of affinity maturation consists of expanding cells, replating at a density of 1E + 06 cells/mL, and performing an additional transient transfection with the AID expression vector 6–7 days prior to cell sorting, at which time cells are stained for antigen binding and surface IgG expression.

1. Treat cells with Accutase or TrypLE according to instructions to release adherent cells, and wash with PBS containing 1% BSA at 4 $^{\circ}$C.

2. Label purified antigen (purchased or produced in-house) with a flow cytometry appropriate amine-reactive fluorescent dye using NHS ester chemistry (e.g., Thermo Fisher), with no more than 1–3 fluorescent tags per protein antigen molecule.

3. Resuspend cells in PBS containing 1% BSA, and incubate at 4 $^{\circ}$C for 30 min with fluorescently tagged antigen at a concentration determined empirically by

prior FACS analysis (see Note 2). Add PE-conjugated goat or mouse anti-human IgG for an additional 30 min at 4 °C.

4. Next, cells are diluted with PBS containing 1% BSA, centri- fuged, and resuspended in PBS (1% BSA) containing propidium iodide to stain dead cells.

5. Cells that express antigen reactive antibody are maintained at 4 °C to slow antigen dissociation and sorted using flow cyto- metry at a rate no greater than 10,000 cells/s. Cells sorted for high antigen/surface IgG based on fluorescence emission, typ- ically 0.1–0.5% of all cells, are pelleted by centrifugation and resuspended at a density of 5E06 cells/mL and plated into a 6-well tissue culture plate containing growth medium and forthwith placed into a 37 °C incubator. During each round of the enrichment process, 2E + 07 cells are typically sorted, yielding ~50–100 K cells for the subsequent round of cell growth, AID transfection, and flow cell sorting.

6. After 3–5 rounds of sorting, the VH and VL sequences are recovered either by next general sequencing methodologies or by sequencing PCR-amplified H and L V-region clones (see Note 3). AID-derived mutations seen in sequencing data often correspond to one or more emerging populations of cells exhi- biting improved fluorescent antigen binding at equivalent levels of surface IgG (Fig. 3).

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2. Typically a concentration ~10–50-fold lower than the K_D of the antibody-antigen interaction. Antigen-binding equilibration periods (2–4 h) or binding competition using a molar excess of unlabeled antigen (following an initial incubation with labeled antigen) may be employed for high-affinity anti- bodies where k_{off} of the antibody-antigen interaction falls below 1E-04/s [12]. Exposure of biotinylated antigen using fluorescently tagged NeutrAvidin or streptavidin (e.g., Dylight 649 Streptavidin), along with fewer or shorter duration cell washing steps to preserve weak binding, is used to increase avidity to low-affinity antibody-antigen interactions.

3. Numerous methods exist for the efficient recombination and screening of libraries in order to identify antibodies with optimized characteristics, such as higher affinity, derived from related clones obtained from phage, yeast, or hybridoma technologies or mutagenesis techniques [10, 21, 22]. It is important, however, to consider other biophysical properties that impact the ability to develop an antibody as a therapeutic such as thermal stability [4], nonspecific binding and clearance [23], and propensity to aggregate in solution [24], as well as solubility, FcRn binding, and dissociation. Antibody mutations arising from in vitro somatic hypermutation, as with in vivo B-cell maturation, fall within CDRs, in particular heavy chain CDR1 and CDR2 at the antigen-contacting residues highlighted in Fig. 1. The position and identity of these mutations can negatively impact properties such as aggregation while simultaneously improving affinity. Up to a third of all somatic hypermutation events fall outside CDR regions and can have a profound impact of stability and manufacturability. Care should be taken to recombine smaller libraries of mutations while simultaneously monitoring affinity, activity, and other pertinent biophysical attributes.

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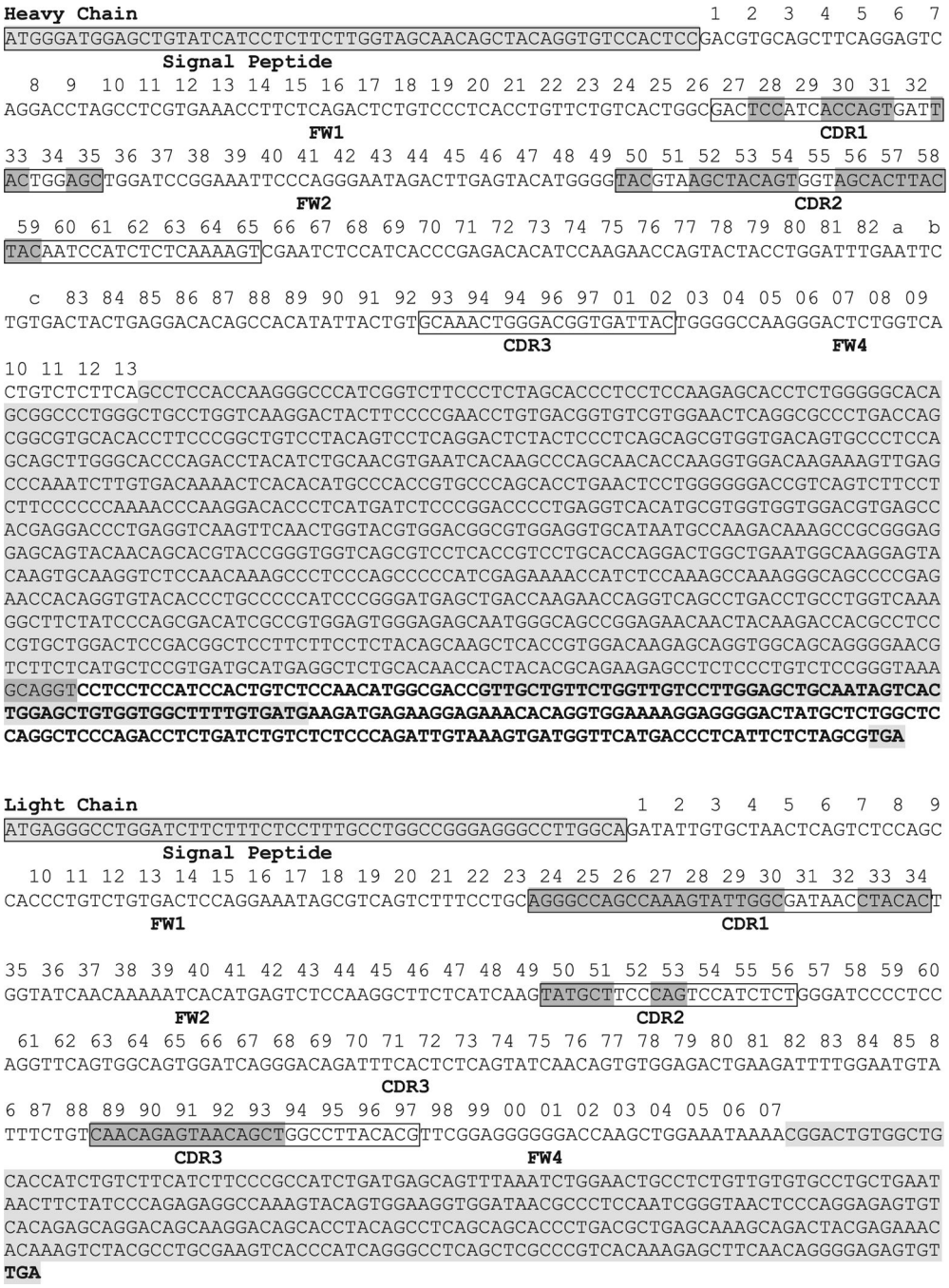

Fig. 1.

Example sequences of heavy chain (above) and a light chain (below) used for surface display are shown. Distinct synthetic signal peptides (gray) support high-level expression and secretion to the cell surface for both the heavy chain and light chain. A mouse variable region of each chain (white) encodes three CDRs (boxed) that make specific side-chain contact (dark gray) with the antigen anchored by framework regions (FW). Labels for regions are shown below the DNA sequence, and Kabat numbering is shown above. These sites are encoded by codons that are commonly altered by AID to generate directed diversity

that may improve binding. Mutations at these Kabat positions are recombined during in vitro SHM to derive affinity- matured variants. Variable regions are joined with full-length human IgG1 and IgK constant regions (gray), and the heavy chains are further joined to a juxta-membrane extracellular (white background/bold text), trans- membrane (gray background/bold text), and cytoplasmic region (white background/bold text) adapted from mouse MHC-1 protein H2kk [19]

ATGGATTCTCTCCTCATGAAGCAGAGAAAGTTTCTCTACCACTTCAAGAACGTCAGATGGGCCAAGGGGA
GACATGAGACGTATCTCTGTTACGTCGTCAAGAGGAGAGACTCAGCCACCTCTTTCTCCCTCGACTTTGG
GCATCTCCGGAACAAGTCTGGGTGTCATGTCGAGCTCCTCTTCCTCCGCTATATCTCAGACTGGGACCTC
GACCCCGGGAGATGCTATAGAGTCACTTGGTTTACCTCTTGGTCCCCCTGTTATGACTGCGCCAGACATG
TCGCCGACTTCCTCAGGGGGTATCCCAATCTCTCCCTCCGCATATTCGCCGCCCGACTCTATTTTTGTGA
GGACAGGAAAGCCGAGCCCGAGGGGCTCAGGAGACTCCACCGGGCCGGGGTCCAGATCGCCATCATGACA
TTTAAGGACTATTTCTATTGTTGGAATACATTTGTCGAGAATCGGGAGAAGACTTTCAAAGCCTGGGAGG
GGCTCCATGAGAATTCTGTCAGACTCTCTAGACAGCTCAGGAGAATT**CTCCTC**CCC**CTC**TATGAGGTCGA
CGAT**CTC**AGAGACGCCTTCCGGACC**CTC**GGG**CTTTGA**

Fig. 2.

An example of a synthetic AID sequence is shown in which potential AID DNA hotspot motifs have been removed using the redundancy of the genetic code, mammalian codon preferences, and other good practice for assembling synthetic gene constructs for expression. The nuclear export sequence (underlined) can be altered at canonical leucine positions to increase AID activity

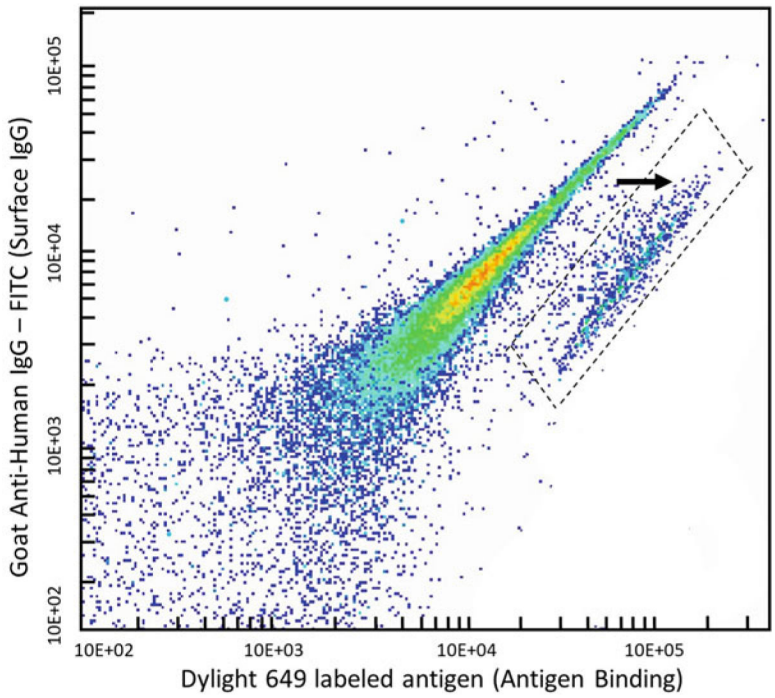

Fig. 3.

An example FACS scatterplot in which cell populations expressing a variant antibody sequence containing AID-derived mutations that impart higher-affinity antigen binding are seen right shifted relative to the parental cell population. Cell staining allows antigen binding as a function of cell surface antibody expression to be carefully quantified. All emergent cell populations are captured for subsequent expansion, sequencing, and additional rounds of AID transfection and flow sorting