Methods xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Antibody humanization by redesign of complementarity-determining region residues proximate to the acceptor framework

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Antibody humanization
Computational protein design
Dead-end elimination

ABSTRACT

Antibodies are key components of the adaptive immune system and are well-established protein therapeutic agents. Typically high-affinity antibodies are obtained by immunization of rodent species that need to be humanized to reduce their immunogenicity. The complementarity-determining regions (CDRs) contain the residues in a defined loop structure that confer antigen binding, which must be retained in the humanized antibody. To design a humanized antibody, we graft the mature murine CDRs onto a germline human acceptor framework. Structural defects due to mismatches at the graft interface can be fixed by mutating some framework residues to murine, or by mutating some residues on the CDRs' backside to human or to a *de novo* designed sequence. The first approach, framework redesign, can yield an antibody with binding better than the CDR graft and one equivalent to the mature murine, and reduced immunogenicity. The second approach, CDR redesign, is presented here as a new approach, yielding an antibody with binding better than the CDR graft, and immunogenicity potentially less than that from framework redesign. Application of both approaches to the humanization of anti-α4 integrin antibody HP1/2 is presented and the concept of the hybrid humanization approach that retains "difficult to match" murine framework amino acids and uses *de novo* CDR design to minimize murine amino acid content and reduce cell-mediated cytotoxicity liabilities is discussed.

1. Introduction

1.1. Motivation

Antibody humanization is an essential engineering step during the preclinical development of monoclonal antibodies. It is aimed at reducing the immunogenicity of monoclonal antibodies derived from non-human sources. The need for humanization approaches was highlighted by the clinical experiences with the first monoclonal antibodies derived from mice using hybridoma technology [1]. These molecules invariably induced human-anti-mouse antibody responses, had short circulation half-life and showed suboptimal interaction with cells of the human effector system [2]. The last two of these shortcomings were largely resolved by the advent of chimerization, a technique whereby mouse variable domains are genetically fused to human constant domains [3]. This technique has not, however, completely resolved the issue of human-anti-mouse antibody responses [4] and, therefore, it has been replaced by approaches in which variable domains of antibodies are humanized as well.

The original technique to humanize antibodies involved "grafting" of murine complementarity-determining regions (CDRs) onto human antibody frameworks [5]. This method introduces defects at the CDR/framework interface and routinely led to reduction of the affinity of the reengineered antibody. It also clearly showed that residues outside of the CDRs play an important role in maintaining optimal antibody/antigen interface. The subset of these residues important for maintaining CDR structures was previously categorized on the basis of early crystal structures of antibodies and was termed "canonical residues" [6]. The next generation of antibody humanization methods built on this understanding by retaining both mouse CDRs and canonical residues in antibody

Abbreviations: CDR, complementarity-determining region; DEE, dead-end elimination; EC50, half maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; FDPB, finite-difference Poisson–Boltzmann; FACS, fluorescence-activated cell sorting; FW4, "framework 4" region (the part of a VH or VL domain after CDR 3); GMEC, global minimum-energy conformation; huKgermB3, human kappa germline B3; huHgermIGHV1-f, human heavy germline IGHV1-f; muHgermV130, murine heavy germline V130; muKgerm19-32, murine kappa germline 19-32.

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http://dx.doi.org/10.1016/j.ymeth.2013.06.024

framework. The most commonly used method developed at Protein Design Labs relied on "backmutating" canonical residues to their parental murine counterparts on the basis of a structural model of variable regions [7]. The list of residues that may require backmutation during the humanization process was subsequently expanded by scientists at Genentech [8–11]. An alternative earlier approach termed "veneering" relied upon substituting surface murine residues outside the CDRs with amino acids commonly found in corresponding positions in human antibodies [12]. This method has failed to gain wide acceptance, potentially due to its resulting large fraction of residual mouse amino acid content and concerns about immunogenic T-cell epitopes that are introduced at multiple junctions between mouse and human sequences.

By the early 2000s, antibody engineering had become a heavily patent-protected and extensively litigated area of biotechnology. Practice of the inventions in antibody humanization area required complex licensing agreements or carried significant royalty burdens. This prompted many biotechnology companies to invest in development of proprietary antibody humanization methods. In this manuscript we describe the research conducted in 2005 at Biogen Idec, when the authors attempted to build on older "CDR grafting" humanization method by using new *in silico* protein design tools and in-house crystal structures of antibodies of interest. This work laid a foundation for the hybrid antibody humanization approaches that are currently in use in Biogen Idec [13,14].

1.2. Antibody of interest

The HP1/2 immunoglobulin or its Fab, whose comprehensive humanization is described herein, binds to its target, α4 integrin, which is a subunit of both α4β1 integrin (also known as very late antigen 4 [VLA-4] or CD49d-CD29) and α4β7 integrin. These integrins, which are expressed on all leukocytes except neutrophils, and on haematopoietic progenitor cells, mediate several homing and adhesive functions [15]. Binding of HP1/2 to α4β1 and α4β7 integrins can prevent each integrin from binding to its cognate receptor, vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressing cell adhesion molecule-1 (MadCAM-1) respectively, which are expressed on endothelium of the central nervous system and of the intestine respectively. (Other ligands of these integrins include osteopontin and fibronectin (CS-1), expressed within the extracellular matrix.) Preventing binding of these integrins to their cognate receptors on endothelium can prevent lymphocyte transmigration out through the blood vessel wall at a "tight junction" in the endothelium. An antibody which binds to these integrins could therefore be an effective treatment for inflammation, for example in multiple sclerosis, Crohn's disease, asthma, or colitis patients.

HP1/2 was chosen from a panel isolated in the lab of Sanchez-Madrid in the late 1980s [16,17]. HP1/2 was found to block VCAM-1 binding to α4β1 integrin but not induce homotypic cell aggregation. The work reported herein took place in 2005 at Biogen Idec, when the authors humanized versions of HP1/2 several ways, including one that retained the complete human framework, with the benefits of new *de novo* design tools and in-house crystal structure of murine HP1/2 Fab. There were no structures available at the time for the target α4, α4β1, nor α4β7 integrins, neither alone nor in complex.

2. Materials and methods

2.1. Isolation and synthesis of antibodies

Total RNA was purified from hybridoma cells, using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized, using First Strand cDNA synthesis Kit (Amersham Biosciences). Heavy and light chain variable regions were amplified by polymerase chain reaction (PCR) using poly(A) primed 1st strand cDNA, 3′ primers CDL-739 for the heavy chain and CDL-738 for the light chain, 5′ primers using degenerate primers specific for most murine antibody gene signal sequences (Molecular Cloning, Cold Spring Harbor Laboratory Press) and Advantage 2 DNA polymerase (Clontech, Mountain View, CA). Cloned heavy and light chain variable regions were ligated into *Escherichia coli* expression vectors with human IgG1 Fab regions and His-tag. Mutagenesis of selected residues was performed using *E. coli* expression vector and QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Fabs were expressed in Rosetta Gami *E. coli*. Bacteria were plasmolized by osmotic shock to release periplasmic fraction. His-tag Fab protein was purified by HisTrap HP Nickel column (Amersham Biosciences) by fast protein liquid chromatography (FPLC). Purified protein was then run on an SDS–PAGE gel to verify the correct molecular weight and purity.

2.2. Humanization

2.2.1. Design of the CDR graft

Antibody humanization began with the analysis of the mature non-human (donor) antibody. The most similar consensus and germline sequences, from the human and also from the donor species, were determined by BLAST search [18]. For each CDR, the Chothia canonical class was determined by the CDR length and by the sequence of the Chothia canonical residues [19]. Special note was taken of any unusual amino acids at canonical residues. Canonical residues are those residues that have been found to be partially conserved among the members of a canonical class, or more generally and usefully, any residue which is predictive or determining for CDR structure. For example, such CDR structure-determining residues can be identified by the methodology of Martin and Thornton [20]. Since this work was done, the canonical clusters of North et al. [21] were published, which we believe should supersede the Chothia canonical classes, because North et al. had a larger, more recent dataset upon which to draw, they used a clustering algorithm to group similar structures, and assignment of a query sequence with unknown structure to one of their clusters by scoring it vs. hidden Markov models of each of their clusters can be more flexible and subtle than Chothia's simple lists of "allowed" amino acids at a few canonical positions. Also since this work was done, the work of Haidar et al. [22] used a different methodology to find CDR structure-determining residues.

All stages of a humanization procedure can be aided by having an accurate three-dimensional structure of the mature donor antibody, alone or in complex with the target antigen. In the case of HP1/2, we had resolved an X-ray crystal structure of the mature murine HP1/2 Fab alone, but no such structure in complex with the target. Had we not had any crystal structure (which is the typical case in practice), the next best thing would have been to create a homology model. An antibody homology model is generally begun by choosing parts of X-ray crystal structures, one per framework chain and one per CDR loop, each chosen by having high sequence similarity to the corresponding part of the mature donor sequence (and, for each CDR loop, the same length). Any of a number of computer programs, such as MODELLER [23], can then stitch together these parts and relax the resulting structure to arrive at a homology model of the mature donor antibody.

The most similar donor-species germline sequence was presumed to be the germline from which the donor immune system developed the mature antibody, thereby making any differences in their CDR sequences. Any differences in their framework (non-CDR) sequences physically remote from the CDRs were presumed to be hypermutations unimportant to target affinity or antibody