Therapeutic applications of superantibodies

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Superantibody technology represents a method to enhance the potency and utility of monoclonal antibodies. The blueprint for superantibody technology is taken from rare naturally occurring superantibodies with unique sequence regions, conferring specific biological functions not detected on most antibodies. In superantibody technology, peptides with specific amino acid sequences are crosslinked to antibodies using affinity-site-specific chemistry. Three types of superantibodies have been engineered: dimerizing superantibodies with enhanced effector potency, superantibodies with the ability to penetrate living cells and superantibodies as vaccines with built-in molecular adjuvant. Collectively, superantibody technology generates a new class of antibodies with higher levels of therapeutic potency.

Mammalian B cells can make antibodies to virtually any substance, including self-molecules and tumor antigens. Antibodies play a central role in humoral immunity by attaching to pathogens and then recruiting effector cells to defend against invading pathogens. Each antibody molecule possesses a variable domain, consisting of a unique amino acid sequence that recognizes different antigens and a constant domain for its effector system [1]. The advent of monoclonal antibodies (mAbs) has made available a source of homogeneous antibodies to almost anything to which antisera can be raised [2–4]. mAb technology can be traced back to a report by Schwaber *et al.* [5]. These authors produced somatic cell hybrids between human B cells and a mouse plasmacytoma and showed that these 'hybridomas' secreted mouse, human or mixed immunoglobulins. Later, Köhler and Milstein [2,6] selected a specific donor B cell population for cell fusion and screened the obtained hybrids for specific antibody reactivity. Thus, mAb technology was invented.

mAbs have revolutionized diagnostic research and development and, as therapeutics, are now rapidly growing segments of the pharmaceutical market. mAbs are used in a wide range of diseases, including virus infection, cancer and autoimmune diseases [7]. The first mAb, muromonab-CD3 (Orthoclone or OKT3), was licensed by the FDA to suppress acute transplant rejection [8]. The most successful mAb so far appears to be Rituximab (Genentech) for the treatment of B cell non-Hodgkin's lymphoma. By 2004, 26 modern antibody-based therapeutic agents have been approved in the European Union and the USA. Approximately 500 of these products are currently in development, ensuring that the number of approved antibody-based products will increase substantially over the coming years [9].

Several factors can reduce therapeutic efficacy: mouse mAbs are immunogenic in humans, inducing a neutralizing response; humanized antibodies have minimal immunogenicity and frequently lower affinity; mAbs are not efficient in penetrating living cells for intracellular targets and mAbs might not

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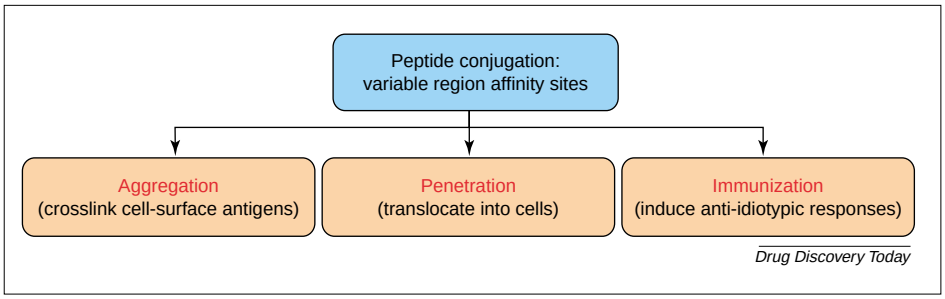

FIGURE 1

Functional advantages of superantibody technology. Functional peptides can be crosslinked to the affinity sites in variable region of mAb. As a result, the formed superantibody acquires functions such as aggregation (self-binding), cell-penetration and vaccinated immunization.

kill target cells. To circumvent these problems, the superantibody technology (SAT) has been recently developed and has shown promising results in enhancing antibody potency both *in vitro* and *in vivo* [10–14].

In this review, we will discuss the origin, effectiveness and applications of SAT in diagnostics and therapy.

Superantibody, nomenclature and classification

SAT is based on site-specific conjugation of antibodies with unique peptides. The term superantibody is derived from the term superantigen, to indicate new features such as self-binding or cell penetration. SAT can be used to obtain three additional properties in the engineered antibody (Figure 1).

mAbs often have insufficient affinity for a given target and it is not practical or cost-effective to generate a new and 'better' mAb. Using SAT, antibody affinity and avidity can be increased. Even in cases where the affinity is acceptable, SAT is able to increase the strength of binding and the utility of the antibody. Crosslinking is a biological signaling mechanism capable of inducing apoptosis [15]. Receptor density or spacing is crucial for crosslinking and is achieved by bivalent antibodies. Polyvalent IgM can induce crosslinking and signaling; however, this class of antibody, even in humanized form, is difficult to scale-up and purify for clinical use. With SAT, the avidity of an IgG antibody can be enhanced.

Being large proteins, antibodies cannot pass through intact cellular or subcellular membranes in living cells. SAT can modify antibodies to penetrate into living cells without harming them. Such cell-penetrating antibodies have been described in auto-immune mice and patients, and peptides responsible for cell and nuclear penetration have been identified [16].

Antibodies themselves can be immunogens, inducing antibody responses termed anti-idiotypes, which can be regulatory or mimic the original antigen. Unfortunately, antibodies are not sufficiently strong immunogens to be used as vaccines and, therefore, they require the use of strong and toxic adjuvants (typically not used in patients) to induce idiotypic mimicry. Anti-idiotypes that mimic antigens have been used as vaccines in animals and humans. Such antibody-based vaccines induce strong responses in mice [17] but they also require a strong adjuvant such as Freund's adjuvant. Equivalent adjuvants for patients are not approved, because of the failure of these vaccines in clinical trials. By conjugating an adjuvant peptide to idiotypic mimicry antibodies, potent vaccines have been developed [14].

Origin of superantibody technology

SAT was not invented in the laboratory but by nature. The origin of this technology comes from the description and study of functional activities associated with antibody V region, a region of the antibody typically not associated with functional activity. SAT is based on several innate biological activities associated with antibody V regions and their re-creation in engineered antibodies.

Functional activities present in the V region are defined by sequences within framework regions and, as such, are selectable and inheritable characteristics (Table 1). Unfortunately, V region functionality, with the exception of the TEPC15 (T15) idiotope, cannot be induced in antibody responses by antigenic stimulation or secondary selection. In addition, V region functional activity, apart from protein A binding, is rarely expressed in mAbs. Thus, such functional activities have been studied, their molecular basis defined and then 'transplanted' into the V regions of normal mAbs.

The first superantibody activity was discovered in mouse T15 plasmacytoma [18] in 1986 [19]. The purified T15 antibodies were found to be able to bind to themselves, showing an autophilic property [20] (Figure 2). Two regions in the T15 V_H domain revealed strong hydropathic complementarities: V_H50–60 was complementary to V_H63–74 [21] (Figure 2a). The sequence region on V_H50–60 covers the complementarity-determining region 2 (CDR2), while the complementary sequence 63–74 is equivalent to one in the framework region 3 (FR3). A 24-mer peptide was synthesized to cover the sequence V_H50–73, which was shown to inhibit self-binding of T15 (Figure 2b). Further studies demonstrated that the autophilic site is present on antibodies with different specificity, suggesting that the autophilic property is conserved through antigen-driven selection [22].

The biological significance and advantage of autophilic antibodies could explain the evolution of the self-binding site in the germline. Antibodies with autophilic properties are directed against non-protein antigens. For example, T15 antibody is directed against C-polysaccharide [18,23] and R24 antibody against a ganglioside [24]. Non-protein antigens cannot be processed and presented to T cells, thus, antigen-driven maturation for these T-independent responses cannot take place. Anti-carbohydrate antibodies are typically IgM, the pentameric structure of which

TABLE 1

Innate V region functional activity

Site	Location	Function	Expression
Protein A binding	FR1 and CDR3 (V_H)	B-cell stimulation	Most Igs
Nucleotide binding	FR1 (V_L) and FR4 (V_H)	unknown	All Igs
Cell penetration	Primarily CDR3 (V_H and V_L)	unknown	Autoantibodies (rare)
Shared idiotype	Invariant framework residues	Network regulation	Autoantibodies and antimicrobial antibodies
Self-binding	CDR2 (V_H) and FR3 (V_H)	Induction of self-binding	Anti-CHO antibodies (rare)

Abbreviations: CDR3, complementarity-determining region 3; CHO, carbohydrate antigen; FR1, framework region 1; FR4, framework region 4.

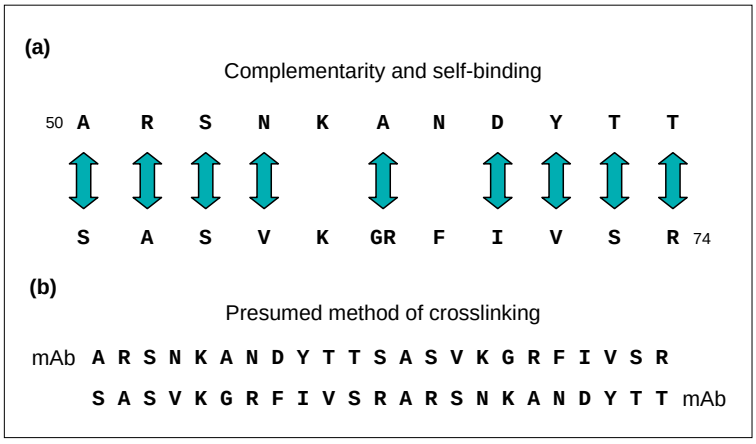

FIGURE 2

Origin, sequence and interaction pattern of T15 peptide. (a) Two hydropathically complementary domains within T15 antibody V_H chain. V_H50–60 is complementary to V_H63–74. Matched amino acid pairs are lined up. **(b)** Designed autophilic peptide from T15 antibody and presumed method of complementarity. The peptide is derived from V_H50–73 in the V_H domain of T15 antibody. Complementary amino acid pairs are lined up.

compensates for low antigen-binding affinity by the increase of the overall avidity.

Experiments by Briles *et al.* [25] showed that autophilic T15 antibody is therapeutically superior compared with non-autophilic antibodies. The self-binding antibody is several fold more potent in protecting immune-deficient mice against pneumococcal pneumonia than non-self-binding antibodies with identical antigen specificity and similar affinity. This observation was confirmed using T15 transgenic mice [26].

Because the autophilic biological activity is endowed in the amino acid sequence of autophilic antibodies, it occurred to us that transplanting these sequences into 'normal' non-autophilic antibodies would allow us to transform such antibodies into 'superantibodies'. In the following sections we describe the engineering of superantibodies and their improved potency.

Generation of superantibodies

Superantibodies are attractive candidates for therapy and, thus, they must meet rigorous criteria of production and safety. Only site-specific chemical conjugation methods can give a batch-consistent product. We have used three site-specific conjugation techniques for covalent attachment of autophilic peptides.

The carbohydrate site on antibodies has been a classical conjugation, producing a fairly reproducible conjugate. The T15 peptide was crosslinked to the oxidized carbohydrate moiety of several antibodies to create superantibodies [10,11]. Two new conjugation methods were developed, based on the discovery of the affinity sites on antibodies: the so-called ATP site [27] and the tryptophan site. Both methods yielded active autophilic antibody conjugates (engineered superantibodies). These conjugates showed enhanced binding to targets and induction of apoptosis in tumors [10,11].

In addition to the use of autophilic peptides, a membrane-penetrating peptide has been employed to create cell-penetrating membrane-translocating antibodies [12,13], and an adjuvant peptide derived from the complement factor C3d has been used to make a molecular adjuvant vaccine based on antibodies [14].

Functional properties of superantibodies

Superantibody dimerization

As a consequence of the therapeutic advantage of autophilic antibodies, it is reasonable to consider that conjugating the autophilic peptide to antibodies with different specificity would endow antibodies with these enhanced therapeutic properties.

Monoclonal mouse and humanized antibodies have been conjugated with the T15 peptide. Conjugated antibodies, as expected, became self-binding and demonstrated enhanced binding to their tumor cell targets. In addition, the T15 conjugates induced more apoptosis in target cells than the original naked antibodies [10,11].

The conversion of a humanized antibody to a self-binding structure with enhanced anti-tumor effects clearly showed the advantage of an autophilic antibody as an effective therapeutic reagent for the treatment of tumor cells. Importantly, efficient induction of apoptosis was also found to depend on crosslinking of tumor target antigens, because the autophilic antibody was proved far more effective than naked antibody, which induced only weak apoptosis [11]. The proposed mechanism of self-binding superantibodies is shown in Figure 3.

It is important to note that autophilic antibodies are non-covalent dimerizing antibodies. The behavior of these autophilic antibodies in solutions of differing ionic strength has been examined [11]. In normal physiological salt solutions, the autophilic antibody behaved as if it were a 'monomeric' Ig, as assessed by HPLC gel filtration. When the ionic strength was reduced, two molecular species were observed, consisting of both monomers and dimers. Re-chromatographing the isolated dimeric fraction, under the same low ionic-strength conditions, again yielded both monomer and dimmer, in the same proportions as in the original fractionation. Thus, the autophilic antibody exists completely as a monomer under physiologic conditions, but as a dynamic equilibrium of monomer and dimer species under low salt conditions. This behavior in solution is an evidence of a multimerization that can occur at target cell surfaces. The non-covalent dimerizing property with low self-binding affinity avoids formation of complexes, under physiological conditions, which have a reduced ability to penetrate tumors and to initiate potential toxic side effects such as fixation of complement. Autophilic conversion was performed with antibodies against the cluster of differentiation-20 (CD-20) and two B cell idiotypes [10,11]. In all cases antibody potency was increased with respect to targeting and apoptotic signal induction.

Superantibody penetration

A limitation of developing therapeutic antibodies is delivery into cells. A class of superantibodies has been developed that bears membrane-transport property. Transmembrane anti-cytoskelton antibodies have been demonstrated to be able to penetrate cell membranes within an hour in culture and to remain active over the period of time that was measured [12]. Furthermore, transmembrane anti-caspase-3 antibodies were shown to penetrate live cells rapidly and increasingly and to inhibit cytotoxin-induced apoptosis, suggesting clinical potential of superantibody penetration [13]. The technology, advantages and applications of the cell-penetrating superantibodies has been reviewed elsewhere [28].

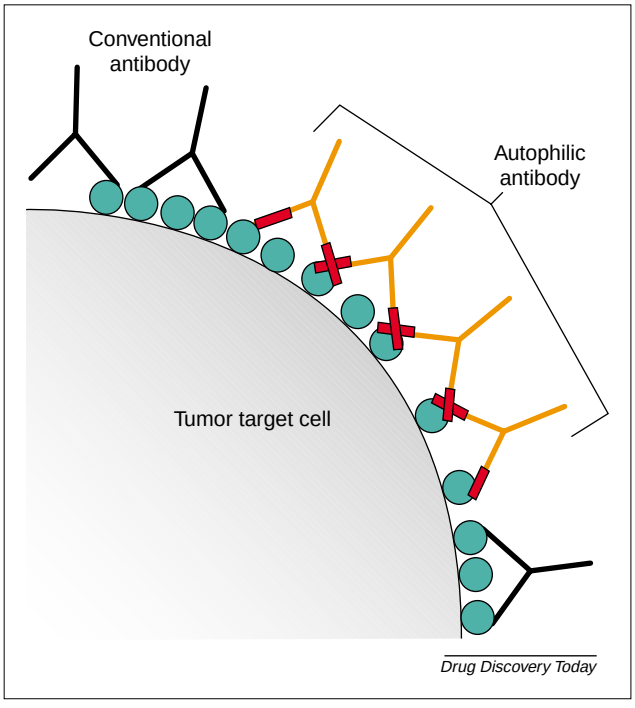

FIGURE 3

Schematic representation of a superantibody binding to a tumor target cell. After a conventional antibody binds to its antigen, it will not trigger intracellular signaling without crosslinking. Superantibody forms aggregation on tumor cell surface and makes it easier to break the threshold for initiation of cell signaling.

Superantibody vaccine

Vaccines composed of antibodies (idiotype vaccines) have given disappointing results in clinical trials because of their inherent low immunogenicity and lack of FDA-approved adjuvants. The identification of adjuvant-type peptides derived from the C3d fragment of complement offered the opportunity to conjugate such peptides to antibodies, creating idiotope vaccines without the need for additional adjuvant. The first C3d conjugated antibody is the well-described idiotype vaccine 3H1 [29], which has been shown to mimic carcinoembryonic antigen (CEA) and to induce in mice antibodies against CEA, but only together with a strong adjuvant. The complement receptor 2 (CR2) binding 16-mer peptide [30] of human C3d was conjugated to the ATP site of 3H1 antibody using photo-activation of 8-azido-adenosine-peptide. Mice immunized with this homologous antibody conjugate produced anti-CEA antibodies that bound to human CEA-positive tumor cells [14], whereas mice immunized with naked 3H1 produced no measurable anti-CEA [14].

Advantages and potential drawbacks of superantibody technology

The advantages of SAT lie in the simplicity of peptide conjugation and affinity-site crosslinking. The photoreactive group on peptides allows for single-step crosslinking that is gentle to the antibody and does not produce harmful end products that require removal. Moreover, there are no restrictions in the choice of peptides for crosslinking, thus permitting endowment of a wide variety of functions to superantibodies. SAT has been applied to produce superantibodies with three different properties: (i) reversible dimerization, (ii) cell membrane penetration, and (iii) molecular adjuvant function. Dimerizing antibodies have higher avidity than monomeric Ig and, thus, they better target and induce crosslinking of cellular receptors. Membrane transporting antibodies can reach targets in living cells and/or organisms in diagnostic and therapeutic applications. Other applications of SAT will include bifunctional antibodies, peptide-mimetic antibodies and biotin–peptide antibodies.

A potential drawback of higher potency and the ability to target even low antigen expressing cells is the possibility of toxic side effects because of low antigen expression in

normal cells. In many cases, antibodies used for tumor therapy identify target antigens that are not restricted to tumor cells alone but are expressed on a restricted number of normal tissues. This is the case for the most successful antibody product, Rituximab, targeting CD-20 on normal and malignant B cells. Although the mechanism of action is still unclear, there is evidence for a role for antibody-dependent cellular cytotoxicity (ADCC) and apoptotic mechanism of action [31], the same mechanism that we can amplify with SAT. Indeed, it is also believed that the antibody operates by removing tumor and normal B cells from the circulation [32]. As regards normal B cells, these can rapidly regenerate from normal stem cells, as it is the case for many normal tissues that are subject to rapid turnover. Thus, toxic effects are expected to be temporary in normal cell populations, for SAT versions, and recovery is expected to be rapid in normal B cells. For those antibodies targeting normal antigens that are not subject to rapid turnover and that are not replaced by stem cell populations an improved targeting and potency would be more problematic. Ultimately, antibodies of superior specificity should be conjoined with the superior potency of SAT.

Enhancing immunoconjugate potency

Several immunoconjugate platforms exist for enhancing potency of naked antibodies. Many of the issues of toxicity and immunogenicity have been, at least partially, addressed. Remaining issues, such as intracellular delivery (in the case of toxins and drugs), tumor retention and sufficient delivered dose of radiation (in the case of radioimmunotherapy products), still represent formidable obstacles to the successful implementation of these platforms with most antibodies. Although current focus has not been on amplifying immunoconjugate potency, several basic properties of SAT conjugates have also been discovered, suggesting their utility. For example, SAT conjugates express higher fluorescence intensity even at saturating concentrations of antibody, as a consequence of more antibodies being bound, of prolonged retention or of a combination of the two. When extrapolated to *in vivo* behavior, these properties are expected to produce enhanced tumor delivery of radiation or other toxic agents. Moreover, there are evidences suggesting that, with some antibody–antigen systems, the crosslinking initiated by self-binding will subsequently trigger internalization. If this can be achieved in most antibody systems, more antibodies could be proved useful in delivering toxic payloads into the cell.

Perspective on the role of superantibody technology in therapeutic applications

SAT can improve therapeutic utility by using these strategies: (i) improving targeting to cells with low antigen expression; (ii) enhancing immunoconjugate potency; and (iii) targeting intracellular functions, thus creating alternatives to drugs, when the development of small molecules is not feasible.

For many mAb-based therapeutic applications, a limiting factor is the degree of antigenic heterogeneity. Even more important to successful tumor therapy is the escape from immunotherapy because of low antigen expression. SAT can restore clinical response in patients and its ability to induce a dynamic crosslinking can be used effectively and antigen-specifically to increase targeting and therapy of even low antigen expressing cells.

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