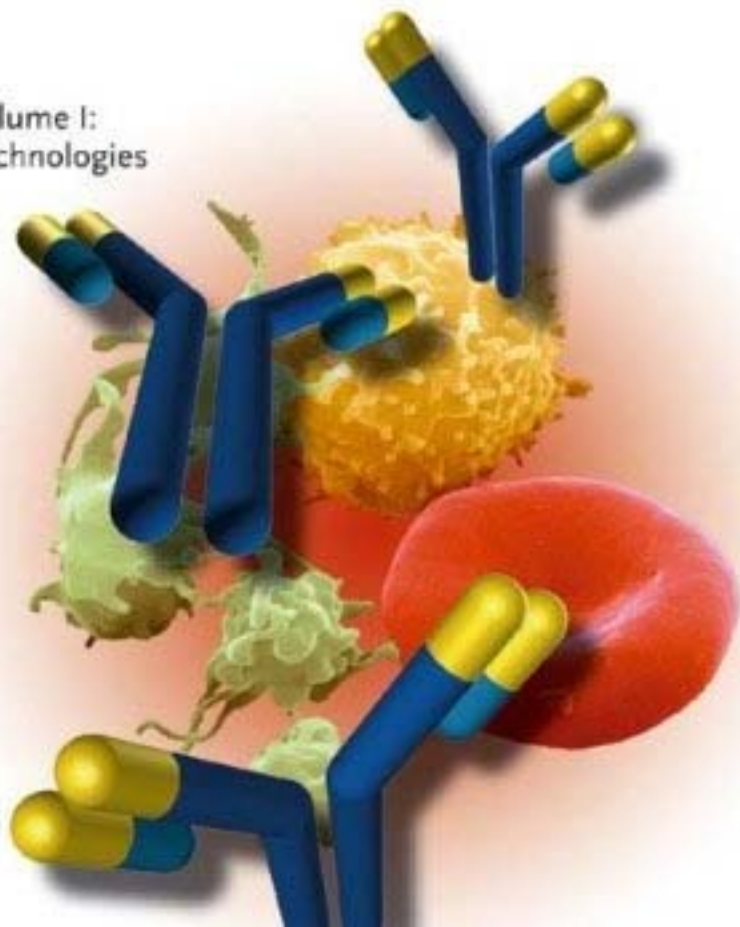


Edited by Stefan Dübel

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Handbook of Therapeutic Antibodies

Volume I:
Technologies



**Handbook of
Therapeutic Antibodies**

*Edited by
Stefan Dübel*

1807–2007 Knowledge for Generations

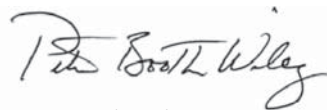
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Volume I

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Stefan Dübel



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hOKT3 γ 1(Ala-Ala) is a humanized IgG1 version of OKT3, in which the amino acids in positions 234 and 235 have been mutated to alanine. Thereby, hOKT3 γ 1(Ala-Ala) was reported to lose complement-activating capacity, Fc γ receptor binding, and mitogenicity. A subsequent phase I study with hOKT3 γ 1(Ala-Ala) demonstrated efficacy similar to that of conventional OKT3 in the treatment of renal allograft rejection with markedly fewer side effects (Woodle et al. 1999). hOKT3 γ 1(Ala-Ala) was also tested in patients with psoriatic arthritis (Utset et al. 2002) or type I diabetes (Herold et al. 2002). In both patient populations, no significant cytokine release was observed, infusion-related toxicity was low and, importantly, these phase II trials suggested clinical efficacy.

Another approach used human IgG2 as a template for the introduction of mutations, because human IgG2 interacts only with Fc γ RIIa, and CD3-directed human IgG2 antibodies required 10- to 100-fold higher antibody concentrations to induce T-cell proliferation. Binding to Fc γ RII receptors was further reduced by two engineered mutations in the constant regions of HuM291 (V234A and G237A) (Cole et al. 1997). As expected, this construct was nonmitogenic *in vitro*. As HuM291 dissociated quickly from cell surface CD3 molecules, only minimal internalization, but sustained signaling by the T-cell receptor was observed. Thereby, HuM291 effectively triggered apoptosis in activated human T cells (Carpenter et al. 2002). In clinical phase I studies in renal allograft or allogeneic bone marrow transplantation patients, the majority of patients did not demonstrate measurable cytokine levels after antibody application, infusion-related toxicity was low, and immunosuppressive activity was observed (Norman et al. 2000; Carpenter et al. 2002).

Protein-engineered antibodies with improved Fc γ receptor binding Clinical observations and animal studies indicate that efficient recruitment of immune effector cells and triggering of cellular effector functions such as ADCC are major mechanisms of action for some therapeutic antibodies (see above). The therapeutic efficacy of these antibodies may be improved by increasing binding of their Fc domains to activating Fc γ Rs. For this strategy, the balance between activating and inhibitory receptors is an important consideration (Nimmerjahn and Ravetch 2005), and optimal effector functions may result from Fc parts with enhanced affinity for activating Fc γ receptors relative to the inhibitory Fc γ RIIb isoform (Clynes et al. 2000; Nimmerjahn and Ravetch 2005). Several different strategies have been followed to identify critical amino acids that could be altered to modify FcR binding and enhance immune effector mechanisms.

In a comprehensive study, all solvent-exposed residues in the Fc part of human IgG1 were individually changed to alanine, and binding to the different Fc γ Rs was analyzed (Shields et al. 2001). These studies identified several groups of mutants that discriminated between binding to Fc γ RI, Fc γ RIIb, and Fc γ RIIIa. Interestingly, several Fc variants, in which two or more amino acids were simultaneously altered to alanine, exhibited additive binding characteristics. For example, the triple Fc mutant S298A/E333A/K334A demonstrated improved binding to Fc γ RIIIa and diminished binding to Fc γ RIIb (Shields et al. 2001).

(Fig. 8.4). Several variants exhibited significant improvements in ADCC with effector cells from either Fc γ RIIIa-V/V or Fc γ RIIIa-F/F homozygous donors. Using this triple mutant with Fc γ RIIIa-F/F donor cells, maximal ADCC levels were increased by more than 100%, and 1–2 logs improvement in potency were observed, as reflected by shifts in the EC₅₀ values to lower concentrations (Shields et al. 2001; Lazar et al. 2006).

In a second approach using a yeast display system (Stavenhagen et al. 2004), mutated human IgG1 Fc regions were screened for altered binding affinity to different Fc γ receptors. For this purpose, a mutant Fc library was generated by error-prone PCR, and the mutated sequences were fused to the Aga2p cell wall protein, which allowed display on the yeast cell wall. This library was screened by soluble tetrameric Fc γ R complexes (Fc γ RIIIa or Fc γ RIIb) and cell sorting. Different screening strategies were applied, combining positive selection and depletion cycles. A variety of Fc mutants with higher affinity for Fc γ RIIIa and enhanced ADCC activity were isolated (see Table 8.3 and Fig. 8.4 for a selection of mutants). Interestingly, several variants with enhanced ADCC activity were identified, in

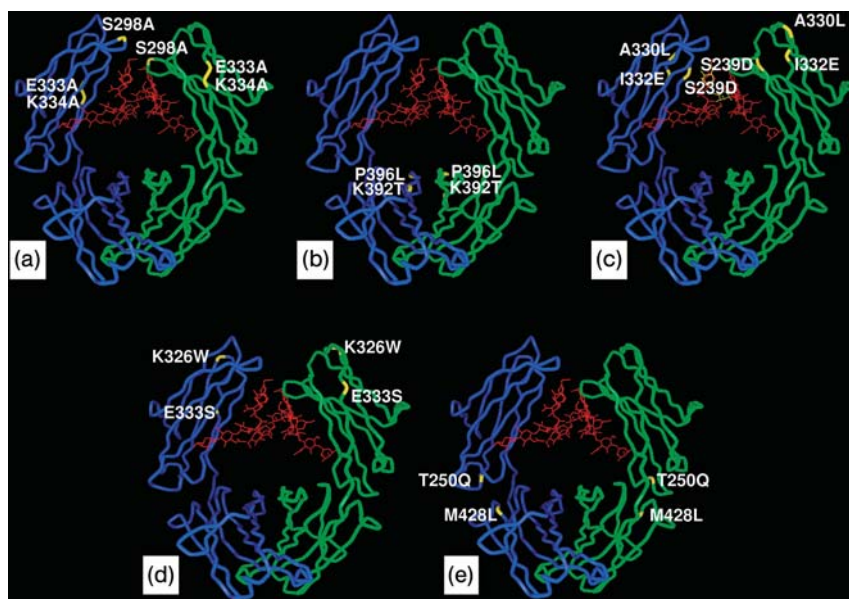


Fig. 8.4 Engineered Fc portions with altered effector functions. (a–c) Enhanced ADCC; (d) altered complement activation; (e) prolonged half-life. (a) S298A/E333A/K334A variant (Shields et al. 2001). (b) K392T/P396L variant (Stavenhagen et al. 2004). (c) S239D/I332E/A330L variant (Lazar et al. 2006). (d) K326W/E333S variant (Idusogie et al.

2001). (e) T250Q/M428L variant (Hinton et al. 2006). Altered amino acid positions resulting in modified effector functions are indicated. Human IgG1 Fc portion: crystal structures adapted from Krapp et al. (2003). Blue and green: C_H2–C_H3 domains; red: carbohydrate; yellow: modified amino acid position.

Table 8.3 Characteristics of selected engineered Fc variants.

Variant	FcγRIIIa binding	FcγRIIb binding	IIIa/IIb ratio	ADCC induction	Fold reduction in EC₅₀ value	Complement activation	Reference
Wildtype	↑	↑	1	↑	—	+	
S298A-E333A-K334A	↑↑	↓	10	↑↑↑	10–100	No data available	Shields et al. 2001
S239D-I332E	↑↑↑	↑↑	4	↑↑↑	100–1000	+	Lazar et al. 2006
S239D-I332E-A330L	↑↑↑↑	↑↑↑	9	↑↑↑	100–1000	—	Lazar et al. 2006
K288N-A330S-P396L	↑↑	↑↑	2	↑↑↑		No data available	Stavenhagen et al. 2004
K392T-P396L	↑↑	↑↑	2	↑↑↑	10–100	No data available	Stavenhagen et al. 2004

IIIa/IIb: Fold FcγRIIIa binding/FcγRIIb binding; EC₅₀: effective concentration 50%; ↑ enhanced activity/binding compared with wildtype; ↓ reduced activity/binding compared with wildtype.

which the mutated amino acids were located outside of the Fc receptor binding region in the C_H3 domain of the antibody (see Fig. 8.4). These data suggest that some mutants increase Fc receptor binding indirectly (e.g. by altering the structural properties of the Fc domain or by influencing the glycosylation of the Fc region) (Stavenhagen et al. 2004).

Lazar and colleagues used computational design algorithms and high-throughput screening to engineer Fc variants with optimized FcγR affinity and specificity (Lazar et al. 2006). When structural information was available (Fc/FcγRIII complex), affinity was directly optimized by designing substitutions that provided more favorable interactions at the Fc/FcγR interface. When structural information was incomplete or lacking (as for the Fc/FcγRIIb complex), calculations provided a set of variants enriched for stability and solubility. A number of engineered Fc variants demonstrated significant enhancements in binding affinity to both human FcγRIIIa-158V and FcγRIIIa-158F alleles along with an improved FcγRIIIa/IIb ratio (Table 8.3; Fig. 8.4). In the context of Campath-1H, both variants S239D/I332E and S239D/I332E/A330L displayed more than two orders of magnitude enhanced ADCC potency compared with wildtype Campath-1H, and were 10-fold more active than the S298A/E333A/K334A triple mutant described by Shields and colleagues. Interestingly, in the rituximab background variant S239D/I332E elicited CDC comparable to wildtype rituximab, while the addition of the A330L mutation ablated CDC. Thus, the set of S239D/I332E and S239D/I332E/A330L variants provides the option for enhancing ADCC with or without triggering CDC as additional mechanism of action. To demonstrate the superior cytotoxic potential of the Fc variants *in vivo*, B-cell depletion using CD20-directed antibodies was analyzed in cynomolgus monkeys. The approximate dose required

for 50% B-cell depletion by wildtype antibody was approximately $10\mu\text{gkg}^{-1}$ per day. For the S239D/I332E variant, a dose of $0.2\mu\text{gkg}^{-1}$ per day was sufficient to achieve 50% depletion – an apparent 50-fold increase in potency (Lazar et al. 2006).

In conclusion, protein-engineered Fc variants demonstrated substantial improvements in Fc receptor-mediated effector functions *in vitro* and in primate models. Whether these types of second-generation antibodies will improve clinical outcomes needs to be evaluated in future clinical trials.

8.2.3

Altered Complement Activation

Since the contribution of complement to the therapeutic efficacy of antibodies is controversial, and since complement activation may contribute to the side effects of antibodies in patients, approaches to either enhance or to diminish complement activation have been proposed. Activation of the classical complement pathway requires binding of the C1q serum protein to the Fc portion of IgG. This C1q binding is completely abolished in Asn297 mutated, aglycosylated IgG, but these mutants also lack Fc receptor binding (see above). Therefore, more complement-specific approaches were required.

Initial studies with a mouse IgG2b antibody mapped the core C1q-binding site to amino acids E318, K320, and K322 (Duncan and Winter 1988). More recent mutational analyses of human IgG1 revealed that the C1q-binding region of human IgG1 is centered around D270, K322, P329, and P331 in the $\text{C}_{\text{H}}2$ domain. Thus, two mutants – D270A and P329A – were particularly ineffective in binding C1q and activating human complement, but still retained some complement-activating capacity at higher complement concentrations. Interestingly, the P329A mutant also demonstrated significantly impaired ADCC activity compared to wildtype, while the ADCC activity of the D270A mutant was similar to the control antibody (Idusogie et al. 2000).

Further studies revealed that amino acids K326 and E333 – located at the edges of the C1q-binding region – profoundly influenced complement activation by IgG1 mutants (Idusogie et al. 2001). Thus, a K326W substitution provided the highest increase in C1q binding (3-fold) and complement-mediated killing (2-fold). In amino acid position 333, the E333S substitution resulted in the highest increase in C1q binding (2-fold) and CDC activity (1.6-fold). The K326W/E333S double mutant demonstrated additive increases in C1q binding (5-fold), but CDC activity was not further increased compared to the single mutants. Interestingly, the K326W and the K326W/E333S mutants were completely inactive in ADCC assays (Fig. 8.4). These observations make them very interesting tools to elucidate the effector mechanism of antibodies *in vivo*, as they were supposed to act only by complement, but not by ADCC.

8.3

Modifying the Pharmacokinetics of Antibodies

8.3.1

Introduction

Studies in rituximab-treated patients have indicated that clinical responses were correlated with favorable pharmacokinetics in patients (Berinstein *et al.* 1998). Therefore, improving the serum half-life of therapeutic antibodies is an attractive approach, which may reduce the amount of required antibodies, and may increase their convenience for patients by prolonging treatment intervals. For most human IgG antibodies, serum half-life is in the range of 3 weeks. Elegant studies have demonstrated that this prolonged half-life of IgG compared with other serum molecules or antibody isotypes is not merely a function of protein size, but requires interaction with a specific Fc receptor. This receptor (FcRn) is a heterodimer of β_2 microglobulin and an HLA class I-related α -chain, which – in humans – is mainly expressed by endothelial cells (Ghetie and Ward 2000). After internalization by fluid phase pinocytosis, IgG is routed to acidic endosomes, where binding to FcRn is believed to occur at low pH. This binding prevents lysosomal degradation of IgG, and triggers sorting and transport to the cell surface, where IgG is released from FcRn at near neutral pH. Thereby, FcRn actively protects IgG from lysosomal degradation, and controls serum half-life of endogenous and exogenous antibodies.

Recent studies have demonstrated that antibody consumption (e.g. by widely expressed and rapidly internalized antigens) may also dramatically influence their serum half-life (Lammerts van Bueren *et al.* 2006; Shih *et al.* 1994). The relative contribution of FcRn binding and target antigen-mediated antibody consumption for the pharmacokinetic profile of therapeutic antibodies is unknown. Whether antibody consumption is amenable to molecular engineering – without impeding antibody efficacy – has not been addressed.

8.3.2

Modifying Binding to FcRn

Analysis of the crystal structure of FcRn with Fc has mapped their interaction site to the interface between C_H2 and C_H3 in IgG molecules (Burmeister *et al.* 1994), while leukocyte Fc receptors bind to IgG in the lower hinge region. This region between C_H2 and C_H3 contains several histidine residues, which may account for the sharply pH-dependent interaction between FcRn and IgG. Considering the potential clinical relevance of this interaction, approaches to modify binding of IgG to FcRn were obvious. However, considering the biology of FcRn, mutations should probably not interfere with the pH dependency of binding.

Initial mutagenesis studies identified 10 human IgG1 mutants with higher affinity toward murine and human FcRn at pH 6.0. However, these mutants

exhibited parallel increases in binding to murine FcRn at pH 7.4, which may explain why their serum half-lives in mice were not prolonged (Dall'Acqua et al. 2002). In another report, modeling the binding of human IgG2 to human FcRn guided the selection of amino acid positions 250, 314, and 428 of the human IgG2 heavy chain for further mutagenesis studies (Hinton et al. 2004). Random mutagenesis identified IgG variants at position 250 and 428 with increased binding to FcRn, while none of the 314 mutants resulted in increased FcRn binding. *In vitro*, the optimal mutants – T250Q, M428L, and T250Q/M428L – demonstrated approximately 4-, 8-, or 27-fold higher binding to FcRn at pH 6.0. In rhesus monkeys, the M428L and the T250Q/M428L mutants showed an approx. 2-fold increase in serum half-lives compared with the wildtype antibody. Furthermore, both amino acid substitutions proved to be transferrable to human IgG1 in a recent report, since amino acids 250 and 428 are conserved between the four human IgG isotypes. The serum half-life in rhesus monkeys was prolonged by 2.5-fold. It may be expected that these mutations also improve the pharmacokinetics of IgG isotypes in humans, provided that the data from rhesus monkeys can be transferred, and assuming that these mutations do not increase the immunogenicity in clinical studies (Hinton et al. 2006) (Fig. 8.4).

8.4

Summary and Conclusions

Despite considerable efforts to elucidate the mechanisms of action for therapeutic monoclonal antibodies, our current understanding of these mechanisms is still rather incomplete. Furthermore, the contribution of individual mechanisms may significantly vary between different target antigens, but also between antibodies targeting the same molecule. At present, it is not determined which preclinical assay will optimally predict clinical responses in patients, but with more antibodies coming along the line, our knowledge about relevant mechanisms will certainly increase. Meanwhile, molecular engineering will provide solid platforms for second-generation antibodies, which allow particular aspects of therapeutic antibodies to be selectively improved. Hopefully, these novel reagents will then translate into further improvements in antibody therapy.

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