Abstract
Monoclonal antibodies are increasingly being used as therapeutic agents in a wide range of indications, including oncology, inflammation and infectious disease. In most cases the basis of the therapeutic function is the high degree of specificity and affinity the antibody-based drug has for its target antigen. However, the mechanism of action (MOA), the way the drug takes advantage of this specificity to mediate a therapeutic effect, varies considerably from drug to drug. Three basic potential categories of MOAs exist: antagonists, agonists and specific delivery mechanisms to target an active function to a particular cell type. The latter functions include selective cell killing, based on Fc-mediated events, recruitment of effector cells, and drug or radioisotope delivery. The majority of these mechanisms are not necessarily optimally mediated by an IgG structure; clearly, in the case of antibody-dependent cellular cytotoxicity or complement-mediated lysis, Fc is required. However, Fab fragments (the fragment comprising one antigen-binding arm of the Y-shaped IgG molecule) can be formatted to mediate most mechanisms and have the advantage that valency and half-life can be controlled to simplify the drug and address only the mechanism required. Moreover, Fab fragments can be produced in microbial expression systems which address manufacturing issues such as scale of supply and cost of goods.

Therapeutic antibody structure and function
Fab' fragments (Fab fragments that include part of the antibody hinge region), used in the univalent form, are applicable particularly to antagonist MOAs. For example, neutralization of cytokines such as interleukin 1β can be achieved using an Fab' with equal potency to that seen with a bivalent IgG. Binding of the two sites of an IgG molecule to molecules such as cytokines appears to be independent, thus univalent fragments are not compromised. Figure 1 shows the relative potency of a univalent Fab' fragment and the bivalent parent IgG antibody in an interleukin 1-neutralization assay. In this system valency conferred no advantage on potency; equivalent neutralization of the interleukin 1β was demonstrated by identical binding sites expressed either as univalent Fab' fragments or as bivalent whole IgG antibodies. The first generation of therapeutic antibodies, which neutralized cytokine function, featured whole IgG molecules as chimaeric, humanized or human antibodies. Modified univalentFab' fragments now offer an alternative approach to the generation of therapeutic reagents with equivalent neutralization potency to whole IgG and which are amenable to large-scale microbial manufacture.

If the therapeutic antibody is to be used to deliver drug or radioisotope selectively to tumour cells, valency is an important consideration, and can dramatically affect the binding properties of the molecule. Multivalent species built up from univalent Fab's can be generated [1]. Figure 2 (top panel) shows the greatly reduced dissociation rate constant of the trivalent tri-Fab'-maleimide (TFM) construct of the antibody B72.3 compared with the bivalent binding of the IgG parent molecule to a solid-phase tumour-associated antigen, mucin. The two constructs share identical
variable regions, and only differ in their valency. The $k_0$ for the IgG was $2 \times 10^{-5}$ s$^{-1}$, and the $k_0$ for the TFM was $6 \times 10^{-8}$ s$^{-1}$, showing a 3-fold improvement in the off-rate for the trivalent species. Figure 2 (bottom panel) shows the additional benefit of increased valency when targeting the CD33 antigen on HL60 tumour cells. The panel shows the internalization rates observed for identical binding regions of the P67.6 antibody expressed either as the TFM or as an IgG. The P67.6 antibody binds to CD33 on the surface of HL60 cells. Disappearance of surface-bound antibody, together with lack of appearance of shed antibody in the supernatant, was taken as evidence of internalization.

Prolonged retention of the drug at tumour sites and increased rates of internalization into tumour cells are highly desirable characteristics to incorporate into designs when considering the optimal therapeutic for drug delivery to tumour cells.

**Antibody pharmacokinetics**

Human IgG molecules have long circulating half-lives in humans, with $t_{1/2\alpha}$ (distribution-phase half-life) values of 18–22 h and $t_{1/2\beta}$ (terminal-elimination-phase half-life) values of 21–23 days for human IgG1, 2 or 4 [2]. It has been found that the pharmacokinetics of human IgG is unusual in that the half-life varies with concentration; at very high IgG levels the serum half-life is decreased. This observation led to the hypothesis that a receptor-mediated event is responsible for maintaining the long circulating half-life for IgG [3], by binding IgG, preventing degradation and recirculating the antibody back to the plasma. It is now known that this receptor-mediated recycling is mediated by the Fc region of the antibody. It has been found that isolated Fc has a half-life of similar duration to the whole IgG molecule, whereas Fab and F(ab')2 are cleared relatively rapidly. Site-directed mutagenesis studies have located the receptor-binding site to a region between the CH1 and CH2 domains [4,5].

The cellular receptor responsible for maintaining half-life has been identified as the neonatal Fc receptor, FcRn (see [6] for review). Mice deficient in this receptor have reduced plasma IgG levels and clear administered IgG or Fc with an abnormally short half-life [5]. Once internalized into cells, IgG is salvaged from the endosome during acidification through binding to FcRn, protecting the antibody from degradation. The IgG is then recycled to the cell surface where the higher pH leads to dissociation and return of the IgG to the circulation.

The half-lives of Fab' fragments are relatively short compared with IgG. Pharmacokinetic experiments in rats (Figure 3) show that a humanized IgG is relatively long-lived ($t_{1/2\alpha} \approx 5.8$ h, $t_{1/2\beta} \approx 104$ h), whereas a Fab fragment derived from the same whole antibody is cleared much more rapidly ($t_{1/2\alpha} \approx 0.3$ h, $t_{1/2\beta} \approx 23$ h). From these data, the calculated area under the curve value for Fab' is $\approx 3.7\%$ of that of the IgG. If the same Fab' fragment is chemically dimerized via a single hinge cysteine residue using bis-maleimido-hexane to form a di-Fab'-maleimide conjugate, the pharmacokinetic profile is fairly similar to monomeric Fab' ($t_{1/2\alpha} \approx 1.5$ h, $t_{1/2\beta} \approx 11$ h, area under the curve $\approx 4.4\%$, compared with IgG).

These results again demonstrate the presence of a specific mechanism for maintaining serum levels of IgG, resulting in a marked difference in circulating half-life of IgG and Fab' fragments. Of note is the short in vivo half-life of di-Fab', a
The increasing development, marketing and usage of antibody-based drugs in medicine has resulted in the near-saturation of existing manufacturing capacity for mammalian cell expression, the traditional route for whole-antibody production [9]. Alternative expression systems are being sought to relieve this capacity constraint and to reduce production costs. Although not currently amenable to expression of full-length antibodies, microbial expression becomes attractive when considering expression of antibody fragments. The larger size of microbial fermenters, short process times and defined salt media contribute to addressing the capacity and cost constraints inherent in mammalian cell expression. While some success has been reported with expression in yeast [10], *Escherichia coli* is currently the host of choice for production of Fab' and other fragments. Using secretion systems, the heavy and light chains of the Fab' are translocated to the bacterial periplasm as directed by leader sequences. Within the periplasm the native Fab' fragment is formed by folding, assembly, and inter- and intra-disulphide bond formation. Published yields of Fab' expressed in this way have varied, some reporting yields greater than 1 g/l [11], while most report more modest yields. Although different expression vectors and induction regimes will account for some of these differences, the sequence of the variable region of the Fab' itself is also apparently important. Some Fab's accumulate to higher soluble levels within the bacterial periplasm than others, presumably because they fold more efficiently into their correct conformation. Other Fab's accumulate poorly and can be toxic to the expressing cell, perhaps because of the saturation of the cell's secretion/folding apparatus. Approaches to obtain more consistent yields have included attempts to modify the variable-region framework sequence to improve folding efficiency, either by identifying and changing individual residues influencing this efficiency [12] or by 'grafting' the antigen-binding loops on to the framework of another well-expressed Fab' [13]. Generating a high yield of Fab' by periplasmic secretion in *E. coli* requires a combination of engineering variable-region framework expressibility, use of a vector system amenable to optimization of relative expression rates of light/heavy chain, and scalability for high-cell-density fermentation. Using this approach, consistent
purified yields of greater than 500 mg/l have been reached for many Fab’s (A. G. Popplewell, unpublished work), and scale-up to 2000 l has been achieved.

**Antibody fragment modification**

As described above, technology exists to efficiently and economically produce Fab’ fragments in an *E. coli* expression system, and by so doing, to address the manufacturing capacity, cost of goods, product volume and development speed issues that limit the applicability of other antibody expression systems. The production of Fab’ fragments may also be advantageous for those clinical settings where Fc functions are not required or may indeed be undesirable from a product safety perspective. It is clear from numerous studies, however, that Fab’ fragments have very short *in vivo* half-lives. It has also been shown that chemical dimerization (Figure 3) or even trimerization [1] of Fab’ fragments does not significantly increase their serum residence, so this cross-linking does not make Fab’ fragments useful for many therapeutic applications where a long half-life is required.

One of the ways in which half-life deficiencies have been addressed for a number of molecules, including proteins, is through the covalent attachment of polymer molecules such as poly-(ethylene glycol) (PEG) [14,15]. Over the past two decades, PEG has been conjugated to various antibodies via random attachment, typically through lysine residues [16]. When applied to antibody fragments such as Fab’, this modification has almost always led to substantial losses in binding activity, most probably due to the attachment of polymer molecules on residues within or proximal to the antigen-binding region of the antibody. In addition, the products formed from this reaction are heterogeneous in nature, which presents manufacturing issues.

One way to address these issues is through site-specific attachment of PEG. It is possible to produce Fab’ fragments with an engineered hinge region containing a single unpaired cysteine residue [1], and this has allowed the site-specific attachment of PEG through use of thiol-reactive chemistry [17]. By so doing, the antigen-binding activity of the Fab’ fragment is maintained and, as shown in Figure 3, the *in vivo* half-life of a Fab’ fragment conjugated to a single 40 kDa PEG entity is markedly increased compared with unmodified Fab’ and approaches that of whole IgG. Similar effects have been observed in humans, with a half-life of a PEGylated Fab’ of 2 weeks.

The site-specific attachment process also results in a homogeneous, defined product which has manufacturing and regulatory benefits.

In order to address valency issues, it has been possible to make use of a PEG terminating in two thiol-reactive groups in order to form a site-specific PEGylated di-Fab’ (A. P. Chapman, unpublished work). Conjugates of this type are required for applications where divalent binding is important for antibody function. These conjugates have also been found to retain antigen-binding activity. It has also been possible to formulate PEGylated Fab’ fragments to a high concentration, allowing their administration by a subcutaneous route. This has many advantages over intravenous infusion and, together with the half-life provided by PEGylation, these antibody conjugates are amenable to monthly dosing by the patient themselves.

Technology has now been developed to make use of Fab’ fragments therapeutically. These fragments can be produced economically and in large volume in *E. coli* expression systems. Therapeutic entities can then be produced in which the valency of antigen binding can be designed and controlled, and similarly the half-life by PEGylation. Molecules of this type are an exciting advance in the use of antibodies where Fc functionality is not required: from neutralization of cytokines or blockade of ligand–receptor interactions, to delivery of effector molecules, such as cytotoxic drugs, to tumour cells.

**References**

Amino acid sequence requirements in the human IgA1 hinge for cleavage by streptococcal IgA1 proteases

B. W. Senior*, M. R. Batten, M. Kilian† and J. M. Wooff

*Department of Molecular and Cellular Pathology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, U.K. and †Department of Medical Microbiology and Immunology, Bartholin Building, University of Aarhus, DK-8000, Aarhus C, Denmark

Abstract

All the IgA1 proteases of the different pathogenic species of Streptococcus cleave the hinge of the α chain of human IgA1 only at one proline–threonine peptide bond. In order to study the importance of these amino acids for cleavage, several hinge mutant recombinant IgA1 antibodies were constructed. The mutations were found to be without major effect upon the structure or functional abilities of the antibodies. However, they had a major effect upon their sensitivity to cleavage by some of the IgA1 proteases.

Introduction

The ability of some important pathogenic bacteria to breach mucosal immune defences is enhanced by their secretion of IgA1 proteases. These are highly specific enzymes that cleave at a specific Pro–Ser (type 1 enzymes) or Pro–Thr (type 2 enzymes) peptide bond in one half of a duplicated octapeptide found only in the hinge of the α1 polypeptide of IgA1 of humans and higher primates, thereby generating Fab and Fc fragments (see Figure 1). The bacterial pathogens that secrete IgA1 proteases are pathogens of mucosal surfaces and the enzymes are believed to act as virulence factors [1–3].

A number of different pathogenic species of Streptococcus that are associated with meningitis, lobar pneumonia, endocarditis, dental caries and periodontal disease produce IgA1 proteases of a zinc metalloprotease type and all cleave the peptide bond between Pro-227 and Thr-228 (Figure 1) [4–7].

As a preliminary step to designing inhibitors of these enzymes, a number of mutant recombinant IgA1 antibodies bearing other amino acids at positions 227 and 228 were prepared (Figure 1). These were examined for the effect of the amino acid substitutions on the sensitivity of the mutant antibodies to cleavage with the different streptococcal IgA1 proteases.

Effect of point mutations in the hinge region

Although the amino acid substitutions were shown to be without major effect upon the structure and functional properties of the antibodies as judged by Fc α receptor-mediated rosette formation and the induction of respiratory bursts in neutrophils, they had a major effect upon cleavage by some of the IgA1 proteases.

Antibodies P227T and T228P were the most resistant of the mutant antibodies to cleavage by the streptococcal IgA1 proteases. P227T was resistant to cleavage by the proteases of all the Streptococcus sanguis and Streptococcus mitis strains tested and partially resistant to that of Streptococcus oralis. However, it was readily cleaved by the protease of Streptococcus pneumoniae to give an Fab fragment of mass consistent with cleavage at or close to the 227–228 peptide bond. The mass of the fragment was quite different from the Fab fragment arising from cleavage of the