Understanding drug resistance to biologic therapy

Resistance—the true face of biological defiance

Nia Emami-Shahri1 and Thorsten Hagemann1

Abstract

Biological therapeutics are widely used in chronic inflammatory and malignant disease. The underlying mechanisms of treatment failure for these drugs are poorly understood. Resistance to these biological agents and the further subdivision into intrinsic and acquired resistance are not clearly defined. In this review, we explore the current understanding of the mechanisms of action of several biological agents as well as the complex biological processes that underlie resistance. A better understanding of why biologicals fail might help to improve their single or combinational use and will ultimately help to alleviate disease burden more efficiently.

Key words: rheumatoid arthritis, cancer, resistance, cytokines, IFN-α, monoclonal antibodies.

Introduction

Biological therapeutics are widely used in a variety of diseases. This novel class of agents are used in chronic inflammatory diseases, such as RA and Crohn’s disease, and also in cancer therapy. There is a renewed focus on biologicals to provide alternative treatment options to failing conventional therapies, to overcome resistance to the biological agents themselves, and also to improve disease burden while causing minimal side effects. However, with the increased utilization of this type of therapeutics one has to raise the question: how is resistance towards them defined?

There needs to be a clear understanding of intrinsic resistance as opposed to acquired resistance. Intrinsic resistance describes the innate ability of target cells to be unaffected by therapeutic modulation, which is already part of the cell machinery at the genetic level. On the other hand, acquired resistance details the adaptation of target cells to bypass therapeutic modulation, which is achieved after prolonged treatment usually through complex biological processes.

It was thought that cytokine immunotherapies, both purified recombinant cytokines and their respective antagonists, would allow for alternative treatment in a range of inflammatory disorders and tumours. Cytokine research has progressed rapidly and a great deal of research has gone into investigating their therapeutic potential in tumourigenesis. It is now known that they play a key role in both eliminating tumours, through activation of immune effector mechanisms, and promoting carcinogenesis. Of note, cytokines are not only produced by host stromal and immune cells, but also by malignant cells. We are now recognizing the value of cross-talk between normal and malignant cells in the development and maintenance of tumourigenesis.

IFN-α is possibly the agent with the most success in cancer therapy, as its anti-tumour effects have been observed in several haematological malignancies and solid tumours [1]. Similarly, IL-2, with its potent immunomodulatory effects, has been used to treat metastatic melanoma and renal cell carcinoma (RCC) with some success [2, 3]. Some clinical advantages have also been seen with other members of the cytokine family, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α [4–7].

Similarly to cytokine immunotherapy, mAbs have come to play a significant role in the therapy of chronic inflammatory diseases and in malignancies [8, 9]. Dysregulated overproduction of inflammatory cytokines (e.g. IL-6, TNF-α, IL-1β) in those cases has been countered with neutralizing mAbs. In particular, anti-TNF mAbs are still used for the treatment of patients with RA [10]. Other mAbs bind specific antigens on target cells, e.g. B cells in the case of rituximab, which is used in the treatment of both RA and haematological malignancies [11].

This review will focus, as an example, on four separate biological agents: IFN-α, infliximab, rituximab, cetuximab and bevacizumab. We will discuss the data generated to

1Centre for Cancer and Inflammation, Barts Cancer Institute, John Vane Science Centre, London, UK.

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Correspondence to: Thorsten Hagemann, Centre for Cancer and Inflammation, Barts Cancer Institute, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, UK.

E-mail: thagemann@qmul.ac.uk
postulate the mechanisms of action and resistance for each antibody; and, where appropriate, describe any data refuting such hypotheses. For an overview of the biological agents discussed see Table 1.

### IFN-α therapy

IFN-α is a cytokine that is produced in response to viral infections. It binds to its receptor, IFNAR, which is expressed on the surface of several cell types. Activation of IFNAR induces downstream signalling pathways, such as the Janus-activated kinase (JAK)-signal transducers and activators of transcription (STAT) pathway. The resultant gene products allow for the immunomodulatory and anti-proliferative effects of IFN-α [65]. IFN-α has been approved for several malignancies, such as chronic myeloid leukaemia (CML) and hairy cell leukaemia (HCL), and has also been indicated for follicular lymphoma (FL), RCC and malignant melanoma [1].

### Mechanisms of action of IFN-α

IFN-α has been found to have anti-proliferative and pro-apoptotic effects [66-70]. Anti-angiogenic properties of IFN-α are attributed to inhibition of VEGF, IL-8 or fibroblast growth factor (FGF) gene expression [71-73]. IFN-α is implicated in the increase of NK cell activity and Th lymphocytes [74]. It has been suggested that IFN-α increases tumour-specific CD8+ T cells in the peripheral blood as well as increasing tumour-infiltrating T lymphocytes [75, 76].

### Mechanisms of resistance to IFN-α

Several groups have generated IFN-α-resistant cell lines to study resistance mechanisms. In one study, resistance in a cutaneous T-cell lymphoma (CTL) cell line to the anti-proliferative effects of IFN-α was suggested to be due to absence of STAT1 protein or mRNA [12]. Romero-Weaver et al. [25] investigated a human leukaemic cell line and observed that resistance to the apoptotic effects of IFN-α correlated with a loss of STAT2 expression. Yang et al. [13] agree that STAT1 and STAT2 expressions are required for the biological activities of IFN-α, but found that defects in STAT3 activation could also pose as a mechanism of resistance. Another proposed mechanism of resistance is an up-regulation of MAL, which has been observed in resistant cell lines as well as in patients with CTL treated with IFN-α and/or photochemotherapy. MAL is a component of membrane lipid rafts, which play a crucial role in signal transduction [14]. Alterations of other members of the JAK-STAT signalling pathway, such as suppressor of cytokine signalling 1 (SOCS-1) and IFNAR, have also been indicated in IFN-α resistance [15-17].

The current data suggest that acquired resistance to IFN-α, through down-regulation of crucial signalling pathway components, allows malignant cells to avoid its anti-tumour effects. However, epigenetic modifications such as methylation, induction of specific micro-RNAs or post-transcriptional modification by glycosylation, or acetylation are barely examined.

### Anti-TNF-α therapy

Infliximab is a chimeric mAb that binds membrane-bound, as well as soluble, TNF-α. The mAb prevents downstream TNF receptor (TNFR) signalling by neutralizing TNF-α. The elucidation of the role of TNF-α in inflammation provided the rationale for targeting cytokines and led to the generation of anti-cytokine mAb. The subsequent observation that several malignant cell types constitutively produce detectable amounts of TNF-α launched an investigation into the role of the cytokine and infliximab in malignancies (reviewed in full by Balkwill [77]). Infliximab is used as a therapeutic agent for the treatment of RA and a range of other inflammatory diseases [78].

TNF-α is initially synthesized as a membrane-bound form that can then be cleaved by a TNF-α-converting enzyme, which produces the soluble form of the cytokine [79]. TNF-α has a role in both the innate and adaptive immunity. Binding of the cytokine to its receptor activates signalling pathways (e.g. the NF-κB pathway) leading to cell differentiation, proliferation and apoptosis [80].

### Mechanisms of action of infliximab

Infliximab was initially found to reduce the levels of other cytokines and chemokines, such as IL-6, IL-1, IL-8 and GM-CSF [81-83]. Investigators soon elucidated the dominant role of TNF-α in regulating those cytokines. Several other mechanisms of action have been proposed, such as reduced levels of VEGF and decreased angiogenesis [84], reduction of leucocyte infiltration [85] and prevention of cartilage catabolism and bone erosion [86, 87].

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**Table 1** Mechanism of resistance to biological agents

<table>
<thead>
<tr>
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Mechanisms of resistance to infliximab

Patients with RA can exhibit a diffuse cellular infiltrate or lymphocyte aggregates in the synovium. In a prospective study, Klaasen et al. [18] found that the majority of clinical responders to infliximab therapy had lymphocyte aggregates compared with aggregates in less than half of non-responders. This potentially indicates the relative contribution of lymphocytes to infliximab resistance, i.e. the redundancy of targeting essentially one cytokine or pathway.

Another possible mechanism of resistance is single nucleotide polymorphisms (SNPs) in the Toll-like receptor and NF-κB signalling pathways, two crucial regulators of inflammatory and immune responses. In a recent study, 187 SNPs were investigated in these two systems. It was observed that rs7744 (MYD88) and rs11591741 (CHUK) were associated with response to etanercept, another anti-TNF-α agent, but not with infliximab [19]. Further investigations could potentially describe SNPs that confer resistance to infliximab therapy.

In a clinical study of resistant RA, patients receiving infliximab therapy were first classified as responders or non-responders and then the non-responders were further subdivided according to their CRP response. Poor responses were seen in patients where infliximab therapy failed to significantly reduce the CRP level at Week 2 [20]. This observation begs the question whether there are compensatory mechanisms involved after anti-cytokine treatment. Buch et al. [21] described a patient with infliximab-resistant RA. Immunohistochemistry of an arthroscopic biopsy detected lymphotoxin-α (LT-α) and subsequent therapy with the non-specific anti-TNF-α mAb, etanercept (a fusion protein between p75 TNF-R and an Ig backbone), which binds both TNF-α and LT-α, produced clinical remission of the disease [21]. It seems that in a setting where TNF-α is not the dominant cytokine, a type of intrinsic resistance can be seen.

In a clinical study of advanced cancer, Brown et al. [22] observed disease stabilization in 17% of patients treated with infliximab. No baseline TNF-α was detected in the plasma of the responders, whereas half of the non-responders had detectable levels. The authors also observed a negative correlation between baseline chemokine (C-C motif) ligand 2 (CCL2) plasma levels and infliximab response [22]. This was corroborated by two sequential Phase II trials [23]. It is thought that less mAb reaches cell membrane-bound TNF-α due to high circulating levels of the cytokine, which limits the biological efficacy of infliximab.

Both intrinsic and acquired resistance seem to play a role in negating the effects of infliximab. The tumour microenvironment, and lymphocytes in particular, are of interest in this setting.

Anti-CD20 therapy

Rituximab is a chimeric humanized mAb that binds to CD20, the pan-B-cell marker. Rituximab was the first mAb to be ever approved by the US Food and Drug Administration (FDA) for clinical use in the treatment of malignancies. It was initially only approved for use in relapsed indolent lymphoma [88] and has since proven efficacy in the treatment of a wide range of haematological malignancies, such as B-cell lymphomas, small lymphocytic lymphoma, FL and mantle cell lymphoma [89–91]. Randomized clinical trials with RA patients have shown the efficacy of rituximab therapy, which is now given to patients who fail to respond to anti-TNF-α mAbs [11].

CD20 is a specific B-cell differentiation antigen in the form of a transmembrane protein, which is expressed on nearly all B cells and is only down-regulated once they have differentiated into plasma cells [92, 93]. The exact role of CD20 has not been elucidated, which is made evident by the normal B cells seen in CD20-deficient mice [94]; however, it is thought to have an important function in the activation, differentiation and growth of B cells [95, 96].

Mechanisms of action of rituximab

Rituximab has been found to have several mechanisms of action, including complement-dependent cytotoxicity (CDC) [97–99], antibody-dependent cellular cytotoxicity (ADCC) [98–102], phagocytosis and programmed cell death (PCD) [103–106]. The majority of these proposed mechanisms have been investigated in vitro on lymphoma cell lines or primary lymphoma cells, as well as in vivo studies using murine lymphoma models. This makes it difficult to translate these findings into the clinical setting, as the relative contribution of each of these mechanisms is poorly understood. This is in part due to the specific mouse model and anti-CD20 mAb used, and also the possibility that the contribution of each mechanism might be different according to lymphoma subtype [24]. The rationale for targeting CD20 is due to it being a stable antigen and that once the antibody binds, it does not internalize, modulate or shed [26].

Mechanisms of resistance to rituximab

As most of the studies that have investigated mechanisms of resistance to rituximab were in vitro experiments using lymphoma cell lines, it is difficult to translate these findings into clinical practice. Using primary lymphoma cells is a step closer to a tumour setting; however, it is quite a challenge to reproduce the complex interactions of rituximab activity with the target cell. Animal models offer certain advantages, but even those face limitations. This is seen in inter-species differential FcγR expression and function [24].

Studies using generated rituximab-resistant cell lines have found an increased resistance to effector mechanisms, CD20 gene and protein down-regulation, and altered cell signalling, e.g. enhanced NF-κB pathway [27, 28]. This observed antigen loss is supported to some extent by clinical findings of rituximab resistance in relapsed patients [29]. Some groups have also reported the occurrence of CD20-dim relapses in patients treated with rituximab [30]. Several mechanisms of rituximab-induced CD20 loss have been proposed, such as internalization [31] and epigenetic modifications [29].
In a clinical study of FL, Weng and Levy [32] found that patients with low-affinity polymorphisms (158V/V) of FcγRIIA respond less to rituximab therapy compared with patients with high-affinity polymorphisms (158V/F or 158F/F) who then go on to have higher progression-free survival. However, both Farag et al. [33] and Dorman et al. [34] did not find that Fcγ polymorphisms in CCL12 could predict response to rituximab therapy. Of note, innate immune effector cells appear to have an impaired function in CCL patients. It is also critical to recognize that effector mechanisms may vary in importance between B-cell malignancies [35].

Recently, a pre-clinical study using primary FL cells observed an inhibitory effect on rituximab-induced ADCC and NK cell activation when CDC was activated [36]. This was further investigated by depleting complement in a syngeneic mouse model, which enhanced overall therapeutic efficacy as well as rituximab-induced NK cell activation [37]. These observations were supported in a clinical study of FL patients with a C1qA polymorphism, who responded better to rituximab therapy due to low levels of C1q [38].

In an RA study, rituximab therapy in relapsed patients was associated with increased levels of B-cell activating factor (BAFF). BAFF is involved in inhibiting B-cell apoptosis. The authors suggested that these striking increases in BAFF levels in relapsed patients could induce survival and regeneration of B cells found in the synovium [39].

Marked levels of chemokine (C-X-C motif) ligand 12 (CXCL12) have been observed in rheumatoid synovial tissues [40]. It has also been shown that CXCL12 contributes to B-cell resistance to apoptosis as well as plasma cell survival [41]. Plasma cells have even been indicated to migrate towards chemokine gradients, such as CXCL12 [42]. It is believed that these factors could be involved in resistance to rituximab therapy in RA.

Cragg et al. [107] argued that anti-CD20 mAbs could be characterized into Type I and Type II mAbs, based on their differential ability to redistribute CD20 in membrane lipid rafts. The rituximab-like Type I anti-CD20 mAbs can greatly induce complement as they effectively redistribute CD20, whereas Type II mAbs are weak activators of complement but potently induce direct PCD. In line with this observation, second-generation anti-CD20 mAbs are being developed with different mechanisms of action. For example, ofatumumab induces high CDC while ocrelizumab weakly activates CDC but strongly activates ADCC. Moreover, third-generation anti-CD20 mAbs have engineered Fc domains that can improve therapeutic efficacy [108]. In targeting B cells, resistance to therapy appears to be partially due to the type of anti-CD20 mAb used and the specific effector pathways it induces.

Anti-epidermal growth factor receptor therapy

Cetuximab is a chimeric humanized mAb that binds to epidermal growth factor receptor (EGFR), thus blocking ligand interaction. Its high-affinity binding to the receptor prevents EGFR phosphorylation [109]. Cetuximab has been studied both as a monotherapy and in combination with a range of chemotherapeutic agents for the treatment of either head and neck or colorectal cancers (CRCs) [110].

EGFR, also known as ErbB1, is one of four erbB receptor family members that are structurally related. It is a transmembrane protein with an intracellular tyrosine kinase domain. As it was the first erbB receptor that was discovered, it is well characterized (for detailed information on EGFR ligands and expression, see [111, 112]).

Several clinical studies have observed poor prognosis and survival in tumours over-expressing the receptor [113, 114]. Autocrine loops involving EGFR and its ligands have been described to aid tumour survival by playing a role in proliferation, angiogenesis and inhibiting apoptosis [115]. Activation of EGFR through ligand binding allows it to form either hetero- or homodimers, which leads to intracellular tyrosine-kinase activation [116].

Mechanisms of action of cetuximab

Cetuximab has been shown to promote apoptosis by affecting the intracellular balance of Bax and Bcl-2 expression and therefore apoptosis [117–120]. Other mechanisms of action suggested are anti-angiogenic effects [120–122], impact on cell-cycle progression [43, 123] and modulation of the extracellular matrix via modulation of MMP expression [44].

Mechanisms of resistance to cetuximab

Kras mutations have been shown to confer resistance to anti-EGFR mAbs [45]. A cohort study of cetuximab-treated CRC patients investigated their mutational status and found that while wild-type (WT) KRAS status produced a favourable response to treatment, mutated KRAS status proved resistant [45–48]. Of note, a small percentage (<1%) of patients with mutated KRAS might still be able to benefit from cetuximab therapy [48]. KRAS mutations provide a clear indication that patients will not respond to the therapy and also explain the previous clinical data gathered before the importance of the mutational status had been described.

Another molecule downstream of EGFR signalling, B-Raf, has also been extensively investigated as the BRAF V600E oncogenic mutation is found in the majority of cancers. In a recent cohort study, Di Nicolantonio et al. [49] showed that BRAF mutational status in metastatic CRC patients were associated with resistance to cetuximab therapy. All of the patients with WT BRAF responded to therapy, whereas 14% of non-responders carried the BRAF V600E mutation. There is a strong rationale then to combine cetuximab with a B-raf inhibitor, such as sorafenib [49].

PI3KCA mutation has also been associated with cetuximab resistance. EGFR activation triggers the PI3K/Akt signalling pathway, which is ordinarily inhibited by phosphatase and tensin homologue (PTEN). PTEN phosphatase inactivation is thus proposed to affect response to cetuximab therapy. Jhawer et al. [50] found that colon cancer
cells were more resistant to cetuximab if they had PTEN loss or PIK3CA mutation compared with WT controls. In contrast, Prenen et al. [51] found that PIK3CA is not a key to determining resistance to cetuximab therapy in metastatic CRC. Most groups agree that PTEN inactivation affects cetuximab response, whereas there is still discussion on the significance of PIK3CA-associated resistance [52, 53]. SNPs of EGFR, EGF and cyclin D1 have also been implicated in response to cetuximab therapy [54, 124, 125]. The current data suggest that resistance to anti-EGFR therapy is largely due to intrinsic, as opposed to acquired, resistance.

Anti-VEGF therapy

Bevacizumab is a humanized IgG1 mAb with a high affinity and specificity for binding VEGF. Bevacizumab does not bind to any other member of the VEGF family [VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF)], but it does inhibit all isoforms of VEGF [125]. VEGF binds to VEGF receptor-1 (VEGFR1) and VEGF receptor-2 (VEGFR2), two transmembrane tyrosine kinases, which are expressed on vascular endothelial cells. Several Phase III trials have shown the clinical benefit of bevacizumab as a monotherapy or in combination with chemotherapy [127, 128]. VEGF is expressed in a range of human tumours and its expression levels have been shown to correlate with poor prognosis, angiogenesis and vascularization [129].

Mechanisms of action of bevacizumab

Bevacizumab has been implicated to have cytostatic effects on endothelial cells. Benjamin et al. [130] used a mouse glioma xenograft model to show that disruption of VEGF expression caused apoptosis in vessels devoid of pericytes (immature vessels), but not in pericyte-associated vessels. The effects of bevacizumab monotherapy on vessel numbers have not been studied in large clinical trials as obtaining serial biopsies of tumours have proven difficult.

Bevacizumab has also been found to normalize the tumour vascular network. Over-expression of VEGF causes tumour vascular abnormalities, such as hyperpermeability, vessel dilatation and poor perfusion. Addressing these abnormalities could potentially allow for better drug delivery to tumours, as they would be better perfused [131, 132]. Additionally, it has been suggested that bevacizumab might have a direct inhibitory effect on malignant cells [55, 56, 133].

Mechanisms of resistance to bevacizumab

One of the mechanisms of resistance that have been postulated is an increase in pro-angiogenic signalling pathways. Casanovas et al. [57] observed transient tumour stasis and reduced vasculature when blocking VEGF signalling in a genetically engineered mouse model. This was followed by restoration of both tumour growth and vasculature. High expressions of angiopoietin 1, FGF 1 and 2, and ephrin A1 were seen in these relapsed tumours. The authors managed to attenuate the observed relapse with a combination therapy of the mAb and an FGF trap [57]. Other studies have found similar results as well as seen induction of other pro-angiogenic factors, such as IL-8 and CXCL12 [58].

Resistance to anti-VEGF therapy has also been considered in terms of bone marrow-derived cell (BMDC) recruitment. These recruited vascular progenitors and vascular modulators can differentiate into endothelial cells and pericytes as well as release growth factors, cytokines, and MMPs [59]. Groups investigating experimentally induced hypoxia in tissues have observed this increased infiltration of BMDCs and vascular progenitors [60]. Others have shown that by blocking the hypoxia inducible factor-1α (HIF-1α) signalling pathway, the abundant infiltrate was abrogated [61].

A growing body of evidence is implicating that in response to anti-VEGF therapy, tumours are coping by adapting a more invasive phenotype. Rubenstein et al. [62] used a neutralizing anti-VEGF antibody to treat xenografted human glioblastoma cells and, not surprisingly, found decreased tumour vasculature and increased tumour cell apoptosis. However, this switched the phenotype of the tumour to a more aggressive invasiveness and a reliance on vessel co-option; a term used to describe tumour cell survival by association with the host vasculature [62]. Kunkel et al. [63] also describe similar results in an orthotopic intra-cerebral model.

Another proposed resistance mechanism that is gathering interest is the protective effect of pericytes on tumour blood vessels [64]. Anti-VEGF therapy failed to produce a significant regression of blood vessels in a mouse xenograft model. However, an almost 40% regression was achieved by targeting VEGFR2 and the platelet-derived growth factor receptor-β (PDGF-β). The authors further showed that this was achieved by an interference with pericyte-endothelial cell interactions [134]. It is believed that pericytes provide survival signals via the Ang1/TIE-2 pathway to endothelial cells and also stabilize blood vessels. This is further supported by results showing that blood vessels without sufficient pericytes are more sensitive to VEGF-targeted therapy [135]. It appears that the cells of the tumour micro-environment are the main perpetrators of anti-VEGF resistance.

Conclusion

For some of the novel biological therapeutics, we do have an understanding of the reasons why they fail to provide patient benefit. A good example is the definition of WT KRAS vs mutant tumours in CRCs, where the mutational status determines the response to therapies. A reason might be that, for most of the clinical antibodies used, we still lack established biomarkers of response. Little is known about the impact of post-translational modifications on resistance to biologicals or the modification of their action.

Understanding the underlying mechanism in more detail will ultimately impact on the use of biologicals in the clinic and might provide a better understanding of why so many
of the proposed targets are not successful therapeutics. Ongoing research into the aforementioned areas is of continued importance as the major challenge to targeted therapy lies in intrinsic and acquired resistance. It will be important to access data from single agent studies, most likely to come from the use in chronic inflammatory disease. Systems biology might provide a useful tool to analyse in vitro and in vivo/in situ situations. Complex genomic aberrations targeting multiple genes through mutation, changes in copy number and methylation occur in most epithelial tumours and are characteristics of inflammatory conditions resulting in marked rewiring of the signalling networks that determine the success of the treatment. Indeed, the development of high-throughput proteomics technologies that interrogate samples at the DNA, RNA, protein and metabolic levels have not been paralleled by improvements in cell biology approaches to understand the consequences of these changes at a cell to an organism level.

The identification of key resistance mechanisms will hopefully lead to improved outcome with the use of biologicals, which will enable us to refine and enhance their efficacy. This will allow for careful selection of patients for certain therapies, spare patients from ineffective treatment, identify novel biomarkers and potentially give rise to new targets and classes of drugs.

### Rheumatology key messages
- There needs to be a clear understanding of intrinsic resistance as opposed to acquired resistance.
- Identifying clinically relevant factors for resistance will be difficult until mechanisms of action are fully understood.
- Refined mAbs will hopefully improve the outcome for both inflammatory diseases and malignancies.

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