Reumatismo, 2006; 58(2):94-103

RASSEGNA

Biologics targeted at TNF: design, production and challenges

Farmaci biologici anti-TNF: progettazione, produzione e sfide aperte

B. Gatto
Department of Pharmaceutical Sciences, University of Padova

RIASSUNTO

Negli ultimi anni sono stati approvati per l'utilizzo clinico tre antagonisti del TNF sviluppati tramite biotecnologie innovative: questi farmaci hanno profondamente cambiato l'approccio terapeutico alle malattie autoimmuni su base infiammatoria, portando all'attenzione del mercato farmaceutico il potenziale di crescita dei biologici diretti alle citochine.

Lo sviluppo di proteine ricombinanti come farmaci mirati al TNF fu la logica conseguenza della dimostrazione che anticorpi anti-TNF erano in grado di modulare la risposta infiammatoria in modelli animali di artrite reumatoide. I primi prodotti biotecnologici ad essere sviluppati per uso terapeutico furono gli anticorpi monoclonali, ed esistono oggi in terapia due monoclonali specifici per il TNF, uno chimerico e uno completamente umano. In parallelo allo sviluppo dei primi anticorpi terapeutici anti-TNF furono progettati recettori solubili del TNF capaci di legare e neutralizzare l'eccesso di citochina circolante. Il grande successo clinico dei recettori solubili fu realizzato grazie alla progettazione di una nuova proteina ricombinante dimerica, ottenuta tramite fusione della parte extracellulare del recettore umano del TNF con la porzione costante di un'immunoglobulina umana.

Tutti gli antagonisti del TNF approvati in terapia sono stati ottenuti grazie a tecniche di biologia molecolare applicata, e costituiscono un paradigma importante nel campo delle biotecnologie farmaceutiche. Finalità di questa rassegna è di analizzare la progettazione e lo sviluppo dei biologici anti-TNF, descriverne i metodi di produzione biotecnologica e le sfide aperte per il miglioramento di questi prodotti innovativi.

Reumatismo, 2006; 58(2):94-103

INTRODUCTION

Autoimmune diseases affect millions of people worldwide and, with more than 70 distinct types of pathologies associated with autoimmune processes, represent a wide and fast-moving area of research and clinical interest (1). Extensive preclinical research has focused on the regulation of cytokine expression with special interest in rheumatoid arthritis (RA), and this multidisciplinary effort has contributed to elucidate the roles of cytokines in this and other disabling autoimmune diseases. Tumor necrosis factor alpha (TNFα) emerged from these studies as a pivotal regulator of expression of other pro-inflammatory cytokines such as Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (2), thus becoming a key target for therapeutic intervention in a redundant cytokine environment. The term TNF indicates two glycoproteins with high homology (≈30% amino-acid identity) belonging to the TNF superfamily. Human lymphotoxin (LT) was the first cytokine to be purified from a B-lymphoblastoid cell line (3) followed soon by the isolation of a second cytotoxic factor, named TNF or cachectin, from a human myeloid-cell line (4). The binding of TNF to its receptor and its displacement by LT further confirmed the functional homologies between the two proteins (5), and TNF and LT, upon isolation of their c-DNAs (6, 7) were renamed TNFα and TNFβ, respectively.

Indirizzo per la corrispondenza:
Prof.ssa Barbara Gatto
Dip. di Scienze Farmaceutiche
Università di Padova
Via Marzolo 5, 35131 Padova
E-mail: barbara.gatto@unipd.it

TNFα and TNFβ can induce a wide variety of effects on a large number of cell types. Both proteins produce their biological responses by binding to specific receptors expressed on the plasma membrane of TNF responsive cells. TNF receptors (TNF-R) belong to the TNF receptors superfamily (8) and at least two distinct cell surface receptors exist on different cell types, TNF-R1 or p55 and TNF-R2 or p75. A conserved domain distinct from the ligand binding surface is present in the extracellular region of both proteins and mediates the assembly of receptor trimers required for signal transduction (9).

List of Abbreviations

Tumor Necrosis Factor (TNF)
Rheumatoid arthritis (RA)
Interleukin-1 (IL-1)
Interleukin-1 (IL-6)
TNF receptor (TNF-R)
Monoclonal antibodies (Mabs)
Human anti-mouse antibodies (HAMA)
Polymerase Chain Reaction (PCR)
Dihydrofolate reductase (DHFR)
Methotrexate (MTX)
Chinese Hamster Ovary (CHO)
Master cell bank (MCB)
Working cell bank (WCB)
Good Manufacturing Practice (GMP)
Bovine Spongiform Encephalopathy (BSE)
Heavy chain variable regions (V_H)
Light chain variable regions (V_L)
Heavy chain constant regions (C_H)
Light chain constant regions (C_L)
Human Anti Chimeric Antibodies (HACA)
Single Chain Variable Fragment (scFv)
Complementary determining region (CDR)
Anti-adalimumab antibodies (AAA)

DEVELOPMENT OF ANTI-TNF THERAPY

As already mentioned, blocking excess TNF can be therapeutically useful through its cascade effects on other pro-inflammatory cytokines. The first logical step in the development of protein drugs able to neutralize TNF emerged from basic preclinical research: hamster monoclonal antibodies (Mabs) directed at murine TNF were able to reduce inflammation as well as joint damage in animal models of arthritis (10), definitively validating the anti-TNF approach in autoimmune diseases.
However, murine monoclonal antibodies suffer many limitations to their therapeutic exploitation due to their inherent immunogenicity, resulting in the early appearance in patients of human anti-mouse antibodies (HAMA). Only chimeric or fully human antibodies in fact gained approval for the therapy of chronic autoimmune diseases: these drugs, respectively infliximab (Remicade®) and adalimumab (Humira®) represent an important line of well-known anti-TNF biologics with clinical applications.
At the same time of development of monoclonal antibodies as therapeutics, soluble forms of TNF receptors (TNF-Rs) were isolated from human urine (11): these truncated receptors are able to bind TNFα and β (11) and can regulate the biological activity of these cytokines (8). The existence of soluble TNF-R in fluids of RA patients indicated an alternative way to exploit protein drugs as regulators of the activity of TNF *in vivo*: the design of dimeric soluble TNF receptors was another important line of development of biologics in the field, and represents a clever way to mimic a pathway of cytokine control. The final therapeutic product of this research is etanercept (Enbrel®), an engineered soluble receptor of TNF with clinical application in several autoimmune disorders, originally approved in 1998 by the Food & Drug Administration to treat the painful joint swelling and deterioration caused by rheumatoid arthritis.
Both types of anti-TNF biologics, dimeric soluble receptors and monoclonal antibodies, were developed through the power of molecular biology, and protein drugs aimed at TNF represent today one of the most successful story in the field of pharmaceutical biotechnology (12, 13).

DIMERIC SOLUBLE RECEPTORS

The use of TNF-Rs in therapy was postulated in the early nineties, and was pursued with success by groups at Immunex, a biotech firm based in Seattle later acquired by Amgen on grounds of the prospected sales of Enbrel. Researchers at Immunex were able to clone and express genes encoding the human and murine receptors using recombinant DNA technology (14-16), obtaining high yields of the recombinant proteins. Monomeric human receptors for TNF were the initial products developed as drugs, but lack of binding to the cytokine was soon evident. This poor performance was overcome by dimerization of the soluble receptor, which brought an evident increase in binding affinity and biological activity (17, 18). The

homodimer was engineered employing molecular biology techniques to construct a chimeric gene: the TNF-binding extracellular portion of human p75 (TNF-R2) was linked to the DNA coding for the constant domains of human IgG1 (Fc) (19). The clone obtained, consisting in totally human sequences, was introduced in a mammalian cell line for expression of the chimeric fusion protein TNFR-Fc. This protein, which after translation is still monomeric, forms disulfide bridges through the cysteine residues of the Fc portion, yielding the active dimeric soluble receptor, which is secreted in the culture media (20). The chimeric soluble receptor binds with high affinity two TNF molecules, thus sequestering the cytokine in biological fluids. It has excellent *in vivo* activity and the presence of the engineered Fc portion allows, besides dimerization, a longer plasmatic halftime to the protein (19).

Construction of the expression vector for rhuTNFR-Fc (etanercept)

Biotechnological production of recombinant proteins involves several steps, which can be summarized as *a)* the initial cloning of the gene coding for the desired protein into a plasmid, with *b)* subsequent insertion of the expressing vector into a suitable cell to obtain a specific host-vector expression system. Host cells can be a prokaryote or an eukaryote, depending on the features of the final protein product. Production of the active therapeutic protein in mammalian cells achieves lower yields than in *E. coli*, but has the advantage of ensuring fidelity in folding and glycosylation. Transfected cells will use their transcription/translation machinery to express discrete quantities of the cloned recombinant protein. After process development on a pilot scale to optimize yield and quality, the protein is *c)* obtained by large-scale fermentation or cell culture processes. The final step is *d)* the purification and quality control of the recombinant protein.

The production of etanercept follows the paradigm outlined above, but step *a)* required the preliminary design of a cloning strategy to construct the chimeric gene coding for the recombinant human fusion protein rhuTNFR:Fc, later named etanercept (20). This was accomplished by linking different human genes, i.e. the TNF binding sequence of p75 receptor and the Fc portion of human IgG1. The gene for TNFR-2 was derived from a cDNA library of human fibroblast cells cloned into the eukaryotic expression vector pCAV/NOT (15) to obtain the plasmid pCAV/NOT-TNFR, used as source for the DNA sequence of the extracellular region of native TNF-R. A fragment from pCAV/NOT-TNFR, containing the sequence encoding the entire extracellular region of TNF-R, terminating with the Aspartate immediately adjacent the transmembrane region (15) was ligated with a second restriction fragment from plasmid pIXY498 (Fig. 1A). The latter sequence codes for the amino acids of the Fc portion of human IgG1, the domain that will constitute the dimerization frame in the final protein construct. To accomplish correct ligation of these two non-cohesive fragments a linker corresponding to the C-terminal sequence of the truncated TNFR gene and the N-terminal coding of IgG1 was created by Polymerase Chain Reaction (PCR) amplification and employed as detailed in figure 1A.

The two gene fragments and the linker were ligated with T4 ligase as the new gene rhuTNFR:Fc in the commercially available cloning vector Bluescript (Fig. 1A). After transformation of *E. coli* the correct clone was identified, sequenced, and moved into the final plasmid (Fig. 1B), which is suitable for expression in mammalian cells; the resulting pCAV-DHFR-huTNFR-Fc has the whole DNA sequence of rhuTNFR:Fc and several control/selection signals for mammalian cells expression, including the gene coding for dihydrofolate reductase (DHFR), a widely used co-amplification method for CHO mammalian cells made deficient in the expression of genomic DHFR (21). The cells transfected with pCAV-DHFR-huTNFR-Fc were selected initially for the expression of DHFR and then analyzed for the desired rhuTNFR:Fc expression. The highest expressing cultures were subjected to amplification by exposure to increasing concentrations of methotrexate (MTX), transferred to suspension culture and the final selection of the best-producing clone was made based on the level of protein expression under these conditions (21). The sequence of the entire coding region was confirmed by DNA sequencing and the host-vector system thus produced constitutes the master cell bank (MCB). MCBs are strictly regulated for all biotech products with therapeutic applications. They are stored under defined conditions as a collection of ampoules of uniform composition, each containing an aliquot of a single pool of cells derived from the selected protein-expressing cell clone (22). The master cell bank is used for preparation of the working cell bank (WCB) and for the end of production cells from the WCB. The steril-

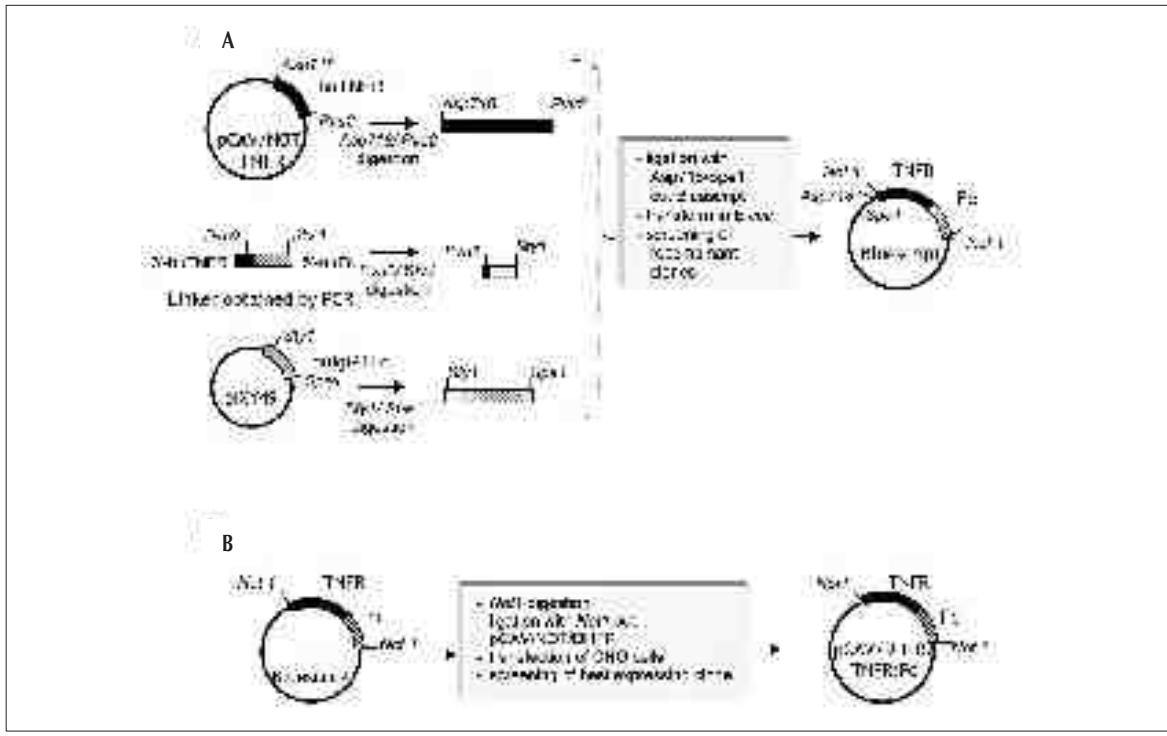

Figure 1 - Construction of the vector coding for etanercept.
A - *Cloning of the gene fusion rhuTNFR:Fc in Bluescript.* The genes coding for the soluble fragment of p75 and Fc were excised by the appropriate plasmids using the indicated restriction enzymes, and were linked together in the plasmid Bluescript using an adaptor created by PCR. After transformation of competent *E.coli* cells, clones were screened and the correct plasmid containing the fusion gene was identified and sequenced. All control/selection signals of the plasmids are omitted for clarity from the drawing.
B - *Cloning of the mammalian expression vector for rhuTNFR:Fc.* The gene coding for rhuTNFR:Fc was excised by the recombinant Bluescript plasmid with the restriction enzyme *Not1* and ligated into the *Not1*-cut expressing vector. pCAV/DHFR/TNFR:Fc was identified after transfection in competent CHO cells and screening in selective media for the best producing clone of the recombinant protein. All control/selection signals of the plasmids are omitted from the drawing.

ity, freedom from mycoplasma, viral safety and retrovirus presence of the MCB, WCB as well as their karyotype, isoenzyme patterns, authenticity, clonality and genetic stability have to be assured as required by Good Manufacturing Practice (GMP) regulations (23). Each WCB is prepared from a single vial of the MCB. New WCBs are tested for the stable production and the identity of the secreted protein.

Production and characterization of etanercept

Recombinant protein expression is achieved by cells grown in different bioreactors according to the scale of production. During cell culture all raw materials from animal origin must not present a risk of BSE and cell cultures are tested for microbial growth, microscopic contamination, endotoxin presence and viability (24). The cell growth kinetics and protein productivity profiles for all bioreactors used in production are tested for extended time periods. In addition, all methods used to control the process, active ingredient and the finished product have to be validated, as well as facilities and equipment according to GMP requirements (25).

Production of etanercept follows these strict rules, and the protein product is tested as required and approved before marketing authorization. As already mentioned, the initial translation product obtained from transfected CHO cells is a single protein chain. Dimerization of rhuTNFR/Fc occurs post-translation through cysteine residues belonging to the Fc portion: two disulphide bonds link monomers to give the dimeric rhuTNFR/Fc, etanercept, which is secreted by producing cells in its active form (20). Etanercept contains 934 amino acids and has an apparent molecular weight of 150 kDa.

Purification of the protein is achieved by a sequential combination of chromatographic/ultrafil-

tration steps, and viral filtration is performed to ensure viral safety. Removal of media components, additives and contaminating virus during purification is also checked. Etanercept is finally characterized using validated physico-chemical, biological and immunological methods for each batch of production. Protein content, pH, uniformity, identity and immunoreactivity are determined as required. The final lyophilized product is also tested for sterility, endotoxin, appearance, residual moisture and reconstitution time. After reconstitution and before each lot is licensed for human use, the final product is tested for color, visible particles and turbidity (26).

MONOCLONAL ANTIBODIES: DESIGN AND PRODUCTION

Monoclonal antibodies, and infliximab in particular, were the first molecules to undergo clinical development (17). Infliximab was introduced to market in 1998 for the treatment of moderate-to-severe Crohn's disease, and is licensed now with indications for several other pathologies (27).

Murine Mabs specific for human TNF were known since the eighties (28-30) and used for research purposes, such as to map epitopes of TNF, to develop immunoassays or to purify the recombinant cytokine (31, 32). The murine monoclonal antibodies derive from hybrid cell lines, known as hybridomas (33): hybridomas are generated by immunization of an animal with a target antigen, then fusing the antibody-producing B cells with mouse tumor cells. The hybridoma cells obtained can be cultured indefinitely to produce monoclonal antibodies, since they combine the immortal growth properties typical of the tumor cell with the antibody production specificity of the original B cell.

The use of murine Mabs as therapeutics in humans was tried with limited success: in particular, a phase I study with an anti-TNF murine Mab in patients with severe septic shock (34) showed early development of human anti-murine antibody response (HAMA) to the treatment, causing decreased effectiveness of continued administration. Chimeric antibodies were hence designed to overcome the HAMA response. Chimeric antibodies are made of a human constant region (C) and part of a variable region (V) of non-human origin, since variable regions contain the antigen binding domains sufficient to determine the antibody specificity. Chimeric constructs in fact retain the high affinity and neutralizing ability of the original intact non-human antibody, while the human IgG1 Fc improves several features of the protein, such as the allogeneic antibody effector function, the circulating serum half-life and the immunogenic response in therapeutic applications.

Development of infliximab

The methods for biotechnological production of chimeric Mabs are well known and rely on the hybridoma technology coupled to genetic engineering techniques (35). The chimeric A2 anti-TNF antibody (cA2), or infliximab, was developed linking the genes of the constant regions of a human IgG1 kappa immunoglobulin human antibody with those of the antigen-binding variable regions cloned from a murine anti-TNFα Mab.

Generation of the original murine anti-TNFα hybridoma was performed by immunization of BALB/c mice with purified recombinant human TNFα (36). The hybridoma cell line A2 established was used for the preparation of the genomic libraries that allowed the isolation of the genes encoding the antigen-binding domains of the murine antibody, i.e. the heavy (V_H) and light (V_L) chain variable regions (36). These DNA segments were joined to the DNA coding for the human constant heavy (C_H) and human constant light (C_L) regions, respectively, to produce chimeric immunoglobulin-encoding genes. This task was accomplished employing plasmid expression vectors carrying functionally complete human C_H and C_L chain sequences, engineered to allow easy insertion of any V_H or V_L chain sequence with appropriate cohesive ends (36).

The two expression vectors carrying the chimeric light and heavy chain genes were inserted in mammalian cells. The recipient cell line used for transfection and production is SP2/0, a myeloma cell that can synthesize, assemble and secrete glycosylated immunoglobulins encoded by transfected genes. The transfected cells are cultured under conditions that permit expression of the incorporated genes either in culture or in the peritoneal cavity of a mouse, and the immunoglobulin chains produced can be recovered as intact antibodies from the culture or from ascites fluid.

SP2/0 cells transfected with infliximab expressing vectors were used to create the master cell bank C168J (27) and WCB for production of the recombinant antibody by continuous perfusion cell culture. After filtration of the supernatants the an-

tibody is purified and tested for microbial contamination, pH, bioburden and endotoxin content. MCB, WCB, facilities and methods are subjected to very strict regulations and validation as discussed for etanercept (25). The different steps of the purification process include affinity and anion chromatography, as well as virus removal steps.
The avidity and epitope specificity of infliximab is derived from the variable region of the original murine A2 Mab. ELISA assays showed cross-competition for TNF between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2 (36). Differently from etanercept, infliximab is specific for TNFα and is not able to neutralize the cytotoxic effects of TNFβ. The chimeric antibody neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner (37).

Development of adalimumab

Many chimeric antibodies still cause adverse events, and, in some patients, they can induce HACA (Human Anti Chimeric Antibodies) response (38). One solution to further reduce the immunogenic responses induced by chimeric antibodies is to remove all mouse-derived sequences, hence to develop fully human monoclonal antibodies. Transgenic mice (39) and phage display techniques (40) are the key technologies that have been widely used to isolate such human antibodies, leading to the approval of adalimumab, a recombinant monoclonal antibody directed to human TNFα, and the first fully human antibody to be approved (41). Adalimumab was developed by Knoll, a division of BASF, and Abbott, and is manufactured by Abbott with the trade name Humira. It is expressed in Chinese Hamster Ovary cells, binds specifically to TN-

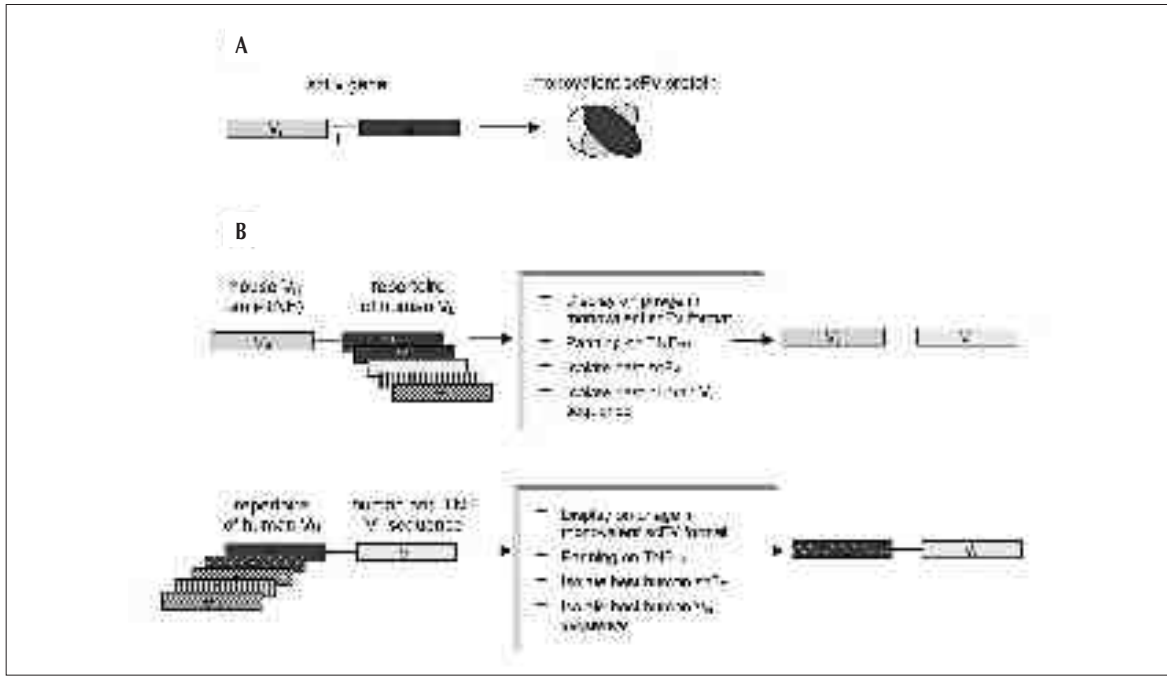

Figure 2 - Isolation of a human antigen binding region by guided selection.
The antigen binding region of a fully human antibody specific for a desired antigen can be obtained by the "guided selection" method (46) employing combinatorial libraries of single chain Fv and phage display technology.
A - *Single chain Fv gene construct and encoded protein*. The genes coding for the heavy (V_H) and light (V_L) variable domains of an antibody can be linked together through a short DNA linker (L) to yield, when expressed, the monomeric scFv
B - *Principle of the "guided selection"*. The gene coding for the mouse heavy variable domain (V_H) specific for a desired antigen (i.e. TNFα) can be linked to the genes coding for the repertoire of human variable light domains (V_L) in scFv format. The combinatorial library obtained is displayed on phages using the phage display technology; the best binder scFv is isolated by panning on TNFα. The gene coding for the related human variable domain (best human V_L sequence) is thus sequenced and linked in scFv format to the genes coding for the repertoire of human heavy variable (V_H) domains. The combinatorial library obtained is again displayed on phages and the best TNFα binder is isolated. The related sequence of best human V_H is determined and the fully human anti-TNF binding region can be obtained after additional rounds of shuffling and CDR mutagenesis.

Fα neutralizing its biological function by blocking interaction with the p55 and p75 TNF receptors. Adalimumab is considered "fully human" in the sense that the coding gene sequences do not contain elements cloned from other animal species. It was obtained by phage display library methodology, a powerful technology to link genotype and phenotype, successfully applied for selecting polypeptides with novel functions (42, 43). In phage display the DNA fragments encoding combinatorial libraries of polypeptides are fused in frame to specific bacteriophage coat protein genes, so that the fusion genes (genotype) are displayed on the phage surfaces. Library members with the desired binding specificities (phenotype) can be isolated *in vitro* by binding to an immobilized receptor, a process called panning. The final step is to determine the sequences of selected high affinity polypeptides, closing in this way the link phenotype-genotype.

Adalimumab was isolated and optimized by Cambridge Antibody Technology originally as D2E7 through guided selection (44) on phage display, using the murine monoclonal antibody MAK195 as the template (45, 46). The murine heavy and light chains were cloned and paired with the repertoire of human light or heavy chains, respectively, in single chain Fv (scFv) format (Fig. 2). At the DNA level the two genes for the variable regions, V_L and V_H, were joined through a synthetic DNA linker using recombinant methods. The gene product will be a single protein chain in which the V_L and V_H regions pair to form the monovalent scFv constituting the antigen-binding portion (Fig. 2A). The mouse-human hybrid scFv libraries resulting after the subcloning were screened in phage display using recombinant human TNFα as the antigen (Fig. 2B), then subjected to cycles of recombination and re-selection to allow generation of diversity and with the aim of improving the binding affinity of the construct for human TNFα (46). By these "mix and match" experiments the preferred human V_L/V_H pair combinations were selected, and further subjected to mutagenesis in the complementary determining region (CDR) by PCR (45, 46), a process resembling the somatic mutation leading to affinity maturation of antibodies during a natural immune response.

The isolated DNA encoding the affinity matured V_H and V_L regions can be converted to full-length immunoglobulin genes by linking them to DNA encoding human constant regions. To do that, the variable region sequences are inserted into mammalian expression vectors already encoding heavy and light chain constant regions (45). The final expression vector for adalimumab hence contains sequences for the variable regions, obtained via phage display, linked to the sequences of the human IgG constant domains.

The fully human antibody is produced in CHO cells transfected with the expression vectors. MCB, WCBs, production and facilities are regulated as required. Adalimumab is purified through several chromatography steps and is subject to low pH treatment and filtration for virus inactivation/removal. Extensive physico-chemical tests, biological and immunological studies have been performed to characterize the final product, an IgG antibody with a total molecular weight of 148 kDa (47).

CHALLENGES

The impact of biotechnology on medicine has been amazing, in terms of basic research, diagnosis and care of diseases with very miserable outlooks just few years ago. The science driving the success of biotech-derived protein drugs has been translated into compounds that have a real impact on the therapy and quality of life of an ever increasing number of patients, who were deemed and doomed as untreatable before the advent of biologics. The success of the three drugs analyzed in this review is reflected by the estimate of 6 billion US Dollars in 2005 for the worldwide pharmaceutical market of TNF antagonists (48).

Manufacturing of these products is changing rapidly and is adapting at a very fast pace to the highly demanding processes of production and quality control of recombinant proteins; the biotech industry is testing new ideas and building sound science on a new area of technological application. The same is true for the national and federal regulators, who are quickly implementing their legislative framework and providing incentives for the biotech production of diagnostics and therapeutics for orphan diseases (49).

Still several challenges trouble the ultimate users of these drugs, patients and their physicians, not to mention the burden of the economic costs of this type of treatment, that may seriously challenge the budget decisions of health care providers. An important issue is the immunogenicity of biotech-derived drugs. Although humanization of recombinant proteins and Mabs was introduced with the intent to reduce HAMA and HACA responses, these

proteins may still elicit the production of specific neutralizing antibodies, with a possible decrease in the efficacy of the drug. The presence of anti-etanercept antibodies is uncommon but was evidenced in RA and psoriasis patients, especially in monotherapy (50, 51). Also the presence of anti-adalimumab antibodies (AAA) was tested in screening assays, with results varying depending on the doses, frequency of administration and concomitant administration of methotrexate, that lowers the incidence of AAA development (47). Antibodies to infliximab have been found both in RA and Crohn's disease patients, and an association between antibodies to infliximab and diminished degree of efficacy has been evidenced (27). The antigenicity of recombinant protein may be related to slight structural differences from the endogenous protein, to incorrect glycosylation, or to amino acids differences in the final product. Also misfolded and denatured proteins are immunogenic, and the correct handling of the protein by the physician, pharmacist and patient will never be stressed with enough emphasis to avoid the formation of aggregates of reconstituted protein in the vial.

Delivery of biotech compounds is an important area of research in pharmaceutical technology. The recent approval of recombinant insulin for inhalation (Exubera®) (52) witnesses the progresses to needle-less forms of delivery for "difficult to deliver" products (53). However, the time when such complex products will be easier to handle and friendlier to the patient is a long way off.

Another issue severely challenging biotech-derived products regards their cost: producing biotech drugs is very expensive, but, as biotech drugs are part of our future, manufacturers are working hard to produce these high molecular weight molecules more cost-effectively. Competition may also play an important role: the first biopharmaceuticals have already begun to come off patent, and, not surprisingly, the market for generic biotech-derived drugs, also called biogenerics or follow-on biologics, is ready to reap the rewards of blockbuster biologics like interferon, human growth hormone, and insulin (54). The European Medicines Agency (EMEA) already passed guidelines for the development and approval of generic biopharmaceuticals (55). However, bringing a biogeneric drug to market is more complicated than for a traditional generic: biotech-derived products are made through complicate manufacturing processes that depend on living organisms. Unlike traditional small-molecule drugs, the direct comparison of one biotech product to another is not good scientific practice. Complex operational and proprietary details of the biotech manufacturing process are central to the definition of the identity of any recombinant protein (56), so that it is not possible to rely on the analytical data generated from one biotech product to support the bioequivalence of a generic version manufactured using another cell line and with different proprietary processes (56). Generic biological products are also harder to characterize than small-molecule drugs, hence harder to prove equivalent to the patented product. Unlike their small-molecule counterparts, biogeneric companies will likely need to conduct their own research and de-

SUMMARY

Several biotech-derived drugs aimed at Tumor Necrosis Factor (TNF) have been licensed in the last years, profoundly changing the therapy of several autoimmune diseases based on inflammation, affecting the life of patients and bringing to the market attention the growth potentials of biologics directed at cytokines.

The proof of principles that led to the design of these compounds dates back from the nineties, when the involvement of TNF in rheumatoid arthritis was proved by the ability of specific anti-TNF proteins to modulate the inflammatory response in animal models. Monoclonal antibodies aimed at neutralizing the excess TNF were developed with therapeutic purposes, and a chimeric and a full human antibody are now approved for several clinical indications. The design of soluble receptors able to bind and neutralize human TNF paralleled the development of antibodies as therapeutics, and the clinical success of these drugs was achieved by the clever design of a novel recombinant dimeric protein, consisting of the extracellular portion of human TNF receptor linked to the constant portion of a human immunoglobulin.

All approved biologics designed to bind and neutralize TNF were obtained through the power of biotechnological methods: the development of these important biopharmaceutical products, their means of production and the challenges they face will be analyzed here in details.

Key words - Anti-TNF biologics, biotech-derived drugs, etanercept, infliximab, adalimumab.

Parole chiave *- Biologici anti-TNF, farmaci biotecnologici, etanercept, infliximab, adalimumab.*

velopment, to set up manufacturing facilities and to conduct expensive clinical trials to demonstrate safety and efficacy. Due to all these costs, biogenerics are predicted to sell for only about 10-20% less than the brand name product (54).
Despite all these tough challenges, biotech-derived products are a reality whose benefit is accepted and valued by society and patients, who should have access to medicines that are safe and effective, and not just cheap.

Acknowledgments
The author is indebted to Prof. Manlio Palumbo and Dr. Giulia Giaretta for helpful suggestions and careful reading of the manuscript.

REFERENCES

1. Ermann J, Fathman CG. Autoimmune diseases: genes, bugs and failed regulation. Nat Immunol 2001; 2: 759-761.
2. Feldmann M. Pathogenesis of arthritis: recent research progress. Nat Immunol 2001; 2: 771-773.
3. Aggarwal BB, Moffat B, Harkins RN. Human lymphotoxin. Production by a lymphoblastoid cell line, purification, and initial characterization. J Biol Chem 1984; 259: 686-691.
4. Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddel DV, Harkins RN. Human tumor necrosis factor. Production, purification, and characterization. J Biol Chem 1985; 260: 2345-2354.
5. Aggarwal BB, Eessalu TE, Hass PE. Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. Nature 1985; 318: 665-667.
6. Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LP, et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 1984; 312: 721-724.
7. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984; 312: 724-729.
8. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003; 3: 745-756.
9. Chan FK-M, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A Domain in TNF Receptors That Mediates Ligand-Independent Receptor Assembly and Signaling. Science 2000; 288: 2351-2354.
10. Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 1992; 89: 9784-9788.
11. Engelmann H, Novick D, Wallach D. Two tumor necrosis factor-binding proteins purified from human urine. Evidence for immunological cross-reactivity with cell surface tumor necrosis factor receptors. J Biol Chem 1990; 265: 1531-1536.
12. Maggon K. Best-selling human medicines 2002-2004. Drug Discov Today 2005; 10: 739-742.
13. Mount C, Featherstone J. Rheumatoid arthritis market. Nat Rev Drug Discov 2005; 4: 11-12.
14. Loetscher H, Pan YC, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W. Molecular cloning and expression of the human 55 Kd tumor necrosis factor receptor. Cell 1990; 61: 351-359.
15. Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 1990; 248: 1019-1023.
16. Goodwin RG, Anderson D, Jerzy R, Davis T, Brannan CI, Copeland NG, Jenkins NA, Smith CA. Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. Mol Cell Biol 1991; 11: 3020-3026.
17. Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol 2002; 2: 364-371.
18. Feldmann M, Maini RN. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. Nat Med 2003; 9: 1245-1250.
19. Goldenberg MM. Etanercept, a novel drug for the treatment of patients with severe, active rheumatoid arthritis. Clin Ther 1999; 21: 75-87.
20. Jacobs C, Smith C; Immunex Corp, assignee. Methods of lowering active TNF-alpha in mammals using tumor necrosis factor receptor. US patent 5,606,690. 1997.
21. Smith CA, Goodwin RG, Beckmann MP; Immunex Corporation, assignee. DNA encoding tumor necrosis factor-alpha and -beta receptors. US patent 5,395,760. 1995.
22. World Health Organization. Requirements for the use of animal cells as in vitro substrates for the production of biologicals (Requirements for Biological Substance N.50). WHO Technical Report Series 1998; 878: 19-53.
23. World Health Organization. Good manufacturing practices for biological products. WHO Technical Report Series 1992; 822: 20-29.
24. World Health Organization. Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology. WHO Technical Report Series 1991; 814: 59-70.
25. Carson KL. Flexibility—the guiding principle for antibody manufacturing. Nat Biotechnol 2005; 23: 1054-1058.
26. EMEA. EPAR on Enbrel; 2005.
27. EMEA. EPAR on Remicade; 2005.
28. Meager A, Parti S, Leung H, Peil E, Mahon B. Preparation and characterization of monoclonal antibodies directed against antigenic determinants of recombinant human tumour necrosis factor (rTNF). Hybridoma 1987; 6: 305-311.

29. Moller A, Emling F, Blohm D, Schlick E, Schollmeier K. Monoclonal antibodies to human tumor necrosis factor alpha: in vitro and in vivo application. Cytokine 1990; 2: 162-169.
30. Liang CM, Liang SM, Jost T, Sand A, Dougas I, Allet B. Production and characterization of monoclonal antibodies against recombinant human tumor necrosis factor/cachectin. Biochem Biophys Res Commun 1986; 137: 847-854.
31. Fendly BM, Toy KJ, Creasey AA, Vitt CR, Larrick JW, Yamamoto R, Lin LS. Murine monoclonal antibodies defining neutralizing epitopes on tumor necrosis factor. Hybridoma 1987; 6: 359-370.
32. Bringman TS, Aggarwal BB. Monoclonal antibodies to human tumor necrosis factors alpha and beta: application for affinity purification, immunoassays, and as structural probes. Hybridoma 1987; 6: 489-507.
33. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256: 495-497.
34. Exley AR, Cohen J, Buurman W, Owen R, Hanson G, Lumley J, Aulakh JM, Bodmer M, Riddell A, Stephens S, et al. Monoclonal antibody to TNF in severe septic shock. Lancet 1990; 335: 1275-1277.
35. Harlow E, Lane D. Antibodies : a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1988.
36. Knight DM, Trinh H, Le J, Siegel S, Shealy D, McDonough M, Scallon B, Moore MA, Vilcek J, Daddona P, et al. Construction and initial characterization of a mouse-human chimeric anti-TNF antibody. Mol Immunol 1993; 30: 1443-1453.
37. Siegel SA, Shealy DJ, Nakada MT, Le J, Woulfe DS, Probert L, Kollias G, Ghrayeb J, Vilcek J, Daddona PE. The mouse/human chimeric monoclonal antibody cA2 neutralizes TNF in vitro and protects transgenic mice from cachexia and TNF lethality in vivo. Cytokine 1995; 7: 15-25.
38. Brekke OH, Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov 2003; 2: 52-62.
39. Lonberg N. Human antibodies from transgenic animals. Nat Biotechnol 2005; 23: 1117-1125.
40. Hoogenboom HR. Selecting and screening recombinant antibody libraries. Nat Biotechnol 2005; 23: 1105-1116.
41. Bain B, Brazil M. Adalimumab. Nat Rev Drug Discov 2003; 2: 693-694.
42. Sidhu S. Phage display in pharmaceutical biotechnology. Curr Opinion Biotechnol 2000; 11: 610-616.
43. Kay BK, Kurakin AV, Hyde-DeRuyscher R. From peptides to drugs via phage display. Drug Discov Today 1998; 3: 370-378.
44. Jespers LS, Roberts A, Mahler SM, Winter G, Hoogenboom HR. Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. Biotechnology (NY) 1994; 12: 899-903.
45. Salfeld J, Allen D, Hoogenboom H, Kaymakcalan Z, Labkovsky B, Mankovich J, McGuinness b, Roberts A, Sakorafas P, Schoenhaut D, Vaughan T, White M, Wilton A; BASF Aktiengesellschaft, assignee. Human antibodies that bind human TNF alpha. US patent 6,090,382. 2000.
46. Osbourn J, Groves M, Vaughan T. From rodent reagents to human therapeutics using antibody guided selection. Methods 2005; 36: 61-68.
47. EMEA. EPAR on Humira; 2005.
48. Moreland LW, Bate G, Kirkpatrick P. Abatacept. Nat Rev Drug Discov 2006; 5: 185-186.
49. Tsang L. Overhauling oversight—European drug legislation. Nat Biotechnol 2005; 23: 1050-1053.
50. Genovese MC, Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, Wasko MC, Moreland LW, Weaver AL, Markenson J, Cannon GW, Spencer-Green G, Finck BK. Etanercept versus methotrexate in patients with early rheumatoid arthritis: two-year radiographic and clinical outcomes. Arthritis Rheum 2002; 46: 1443-1450.
51. Leonardi CL, Powers JL, Matheson RT, Goffe BS, Zitnik R, Wang A, Gottlieb AB, the Etanercept Psoriasis Study Group. Etanercept as Monotherapy in Patients with Psoriasis. N Engl J Med 2003; 349: 2014-2022.
52. News In Brief. Nat Rev Drug Discov 2006; 5: 178-179.
53. Rosen H, Abribat T. The rise and rise of drug delivery. Nat Rev Drug Discov 2005; 4: 381-385.
54. Ainsworth S. Biopharmaceuticals. Chem & Engineering News 2005; 83: 21-29.
55. Committee for medicinal products for human use (CHMP) - EMEA. Guideline on similar biological medicinal products. http://www.emea.eu.int; 2005.
56. Garnick R. Counterpoint: Why biogenerics are a strawman. 2006; 24: 269.