New Cytokines as Potential Drugs

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Preface

When recombinant DNA technology burst forth on the scientific arena in the late 70s and early 80s, one of the first promises that everyone expected to be fulfilled was the treatment of various diseases with the human body's very own arsenal of growth factors and cytokines. The euphoria that accompanied drugs such as Interferon alpha and Interleukin 2 throughout the course of their development was unprecedented. While biotechnology companies mushroomed all over the world and created fortunes overnight on Wall Street, the first pioneers in this area were being faced with pragmatic issues of bringing biologicals to the market. The task of achieving economically feasible levels of recombinant protein expression, revamping manufacturing facilities to meet regulatory standards and struggling to establish norms and rules to regulate the clinical testing were daunting. All of these, however, dealt with issues that could be resolved sooner or later, given perseverance; and almost all of them were, except one – the paradigm for clinical testing of these molecules. In the case of IFNα, e.g., the simple thinking guiding the entire effort was the anti-viral use of these molecules. In the early days of IFNα development no-one suspected that it had multifaceted, pleiotropic, immunological effects on various cell types. Although Interferon α is a successful drug today, it required a Herculean effort to get it to this stage. Several factors stymied earlier efforts at developing some of these drugs which, based on their biology, had a high potential for treating life threatening diseases such as cancer. It soon became apparent that the guiding principle of "more is better", which most oncologists of the day practiced with cytotoxic drugs, did not hold true in the case of cytokines. Cytokines are generally produced in small quantities, sometimes by very specific subsets of cells, in response to a local stimulus or challenge. Additionally, the function of each cytokine is modulated through its binding to a very specific receptor on the surface of selective cells. Except for states of extreme immune or pathological dysregulation, many of these cytokines are rarely seen in measurable quantities in the blood of normal individuals. Early clinical trials established the fact that systemic administration of cytokines needed to be controlled carefully with regard to dose and regimen to avoid side-effects. The side-effects of many of the cytokines such as capillary leak syndrome, flu-like symp-

toms etc. probably result from the fact that the dose of cytokine given by necessity is such that it is now able to affect cells that it otherwise would rarely encounter under normal circumstances.

Despite the hurdles, several cytokines and growth factors have become successful drugs. Some of the most successful drugs such as G-CSF (Neupogen) and erythropoietin (Epogen) are targeted to very specific medical needs and are rather unique in that their functional effects are on a narrow range of cells. This lessens the severity of the side-effects profile and makes the drugs far better tolerated than one that is pleiotropic. A tremendous amount of work has gone into studying the structure-function relationships of cytokines in an effort to identify modifications that could preclude unwanted activities. Elucidation of co-crystal structures of cytokine-receptors has shed further light on the interaction of these molecules. The goal of all these studies is, to one day be able to design small molecule drugs that could be ingested as pills, but would do the work of a complex cytokine molecule. Small peptidic molecules have already been identified, as in the case of erythropoietin. However, until such breakthroughs become realities, the lure of developing a cytokine will continue to excite and challenge entrepreneurs and pharmaceutical companies alike.

One of the major areas of cytokine research that has seen some welcome victories is that of cytokine antagonists. The clinical success of therapies targeted at inhibiting TNFα and IL-4 have promoted monoclonal antibodies and soluble receptors to front runners in the area of therapeutic biologicals. With the feasibility of using fully human monoclonal antibodies, the potential for antigenicity has also diminished, making it possible to position some of these therapies for chronic use. The clinical data clearly shows that inhibition of a single cytokine, even a pleiotropic one, has very specific effects on certain aspects of the disease and overall progression of the disease.

That we will continue to see a profusion of cytokines, growth factors and other novel molecules is almost guaranteed by the large scale "DNA mining" that is under way with the public and private cDNA sequence databases. The challenge here, of course, would be in putting "reverse biology" into real-life practice. The challenge is not lost upon entrepreneurs and several brilliant ideas have formed the basis for the generation of a new mini-industry which caters specifically to "functional genomics" – basically elucidating the function of genes about which nothing more than which cells or tissues produce it might be known. Traditionally, the identification of a factor has always started from identification of its activity by scientists. This meant that the starting point was the availability of an assay. Such an assay could be used to follow purification protocols. If lucky, perhaps some purified protein would be available which could be sequenced. This protein sequence would then be the entry point into screening a library for a cDNA clone. The other route would be to identify the cDNA clone through expression cloning. Once the clone was available, it opened up the door to large scale production, full biological char-

acterisation and perhaps commercial development. Today, however, the full length clone can be put together from the DNA databases, but the formidable task of identifying the relevant functions remains the major time-consuming hurdle. The use of transgenic and knockout mice will probably play a major role in elucidating the function of all these new and exciting molecules.

In putting together this book, we have kept all these emerging trends in mind. Hence, we have here descriptions of cytokines such as FLT3 ligand and Interleukin 10, which are well into the final phases of clinical testing. On the other hand, we also have a chapter on IL-18. Identified in 1997, IL-18 is a sober reminder of the fact that multiple cytokines carry out related tasks. It is a cytokine that partners with IL-12 to propel T cells into the TH1 pathway. Coming behind IL-12, which had already been established as the cytokine involved in the induction of the TH1 response, IL-18's involvement in the TH1 differentiation pathway came as somewhat of a surprise. However, experiments carried out by several different groups have clearly established it as an equally important cytokine in the T cell differentiation pathway. In this case, both the cytokine and its antagonist are likely to be useful drugs. In addition, the new-wave cytokines such as those mined from the sequence database have not been ignored. Keratinocyte growth Factor 2 is a perfect example of this ilk. Identified from the Human Genome Sciences database as a homolog of KGF1, this molecule is among the first to have been derived directly through sequence identification.

To round off the developments in the area of cytokine research, we could not ignore the ever-growing family of the chemotactic cytokines, the chemokines. The identification of CCR5 and CXCR4 as receptors of HIV focused the world's attention on these molecules. The fact that chemokine receptors are G protein-coupled receptors (GPCR) was not lost on the pharmaceutical companies. With the track record of approximately 60% of all approved drugs being GPCR antagonists or agonists, the potential for obtaining small molecule drugs to inhibit HIV entry is enormous. As an added advantage, the identification of several novel receptor ligand pairs and their participation in the trafficking of specific cell types has opened up the possibility of treating diseases marked by inflammatory cellular infiltrates such as asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, etc.

In the coming decade, the impact of genomics will surely be felt in the area of cytokine research. The potential exists for the discovery and development of several new drugs in this category. It is also likely that a number of cytokine antagonists in the form of monoclonals and soluble receptors will be tested clinically. As such, this area continues to hold forth the promise of ground-breaking therapies in multiple diseases.

Contents

List of contributors vii

Preface ix

Francis M. Cuss
Interleukin 5 1

Satwant Narula, Sidney Smith and Paul Grint
Interleukin 10 15

William W. Cruikshank, Hardy Kornfeld and David M. Center
Interleukin 16 31

François Fossiez, Jean-François Nicolas and Serge Lebecque
Interleukin 17 53

Masashi Kurimoto
Interleukin 18 59

Hilary J. McKenna
FLT3 ligand 81

Pablo A. Jimenez, Dale Greenwalt, Donna L. Mendrick, Mark A. Rampy, Jeffrey Su, Kam H. Leung and Kevin M. Connolly
Keratinocyte growth factor-2 101

Sergio A. Lira, Paul J. Zavodny and Daniel Lundell
Chemokines 121

Index 139

Interleukin 5

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Introduction

Most chronic inflammatory diseases have been treated for several years now with steroids and anti-inflammatory drugs. While these treatment have certainly been effective the broad pharmacological effects of these treatments can lead to side effects. However, the advances made in the area of dissecting the pathogenesis of chronic diseases at the cellular and molecular level has, for the first time, provided the opportunity to target cell-specific molecules. Asthma, in particular, is a chronic inflammatory disease that has been studied in great detail. It is now well-accepted that the number of many types of inflammatory cells are increased in asthma and could be responsible for the tissue destruction and clinical sequelae. Eosinophils, for instance, by virtue of their large numbers in pulmonary inflammatory infiltrates and their destructive potential probably play a very significant role. Over the last ten to fifteen years, the understanding of T cell biology has increased dramatically and many inflammatory diseases such as asthma are being seen as having a significant pathogenic component at the level of T cell reactivity. Asthma is characterized by a polarized T helper cell response, and a number of studies both in animal models and human subjects indicate that the predominant T helper cell involved in allergic disease is the TH2 cell type. T helper subsets can be divided into two major subtypes, the TH1 subtype being primarily responsible for cell mediated immunity and the TH2 for the humoral response including IgE. TH1 cells can be identified based on the release of IL-2 and IFNγ and the TH2 subtype on the secretion of IL-4, IL-5 and IL-10.

While IL-4 is recognized as the essential cytokine for generating and maintaining a TH2 response, IL-5 has a pivotal but narrow functional effect on eosinophils and as such is considered particularly suitable as a therapeutic target. There is a great deal of circumstantial evidence for the pivotal role of interleukin 5 (IL-5) in asthma and other inflammatory disorders where eosinophils are prominent. This evidence includes studies in animal models, measurements of IL-5 in human and animal tis-

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sue, and the identification of the essential role of IL-5 in the maturation of eosinophil precursor cells to eosinophils in bone marrow. Eosinophils are seen to accumulate in some inflamed tissue, such as the lungs of patients with asthma, the nasal cavity of patients with allergic rhinitis, and the skin of patients with atopic dermatitis.

One of the major attractions of IL-5 inhibition as a target for therapeutic intervention is the fact that in humans the IL-5 receptor is limited to two cell types, eosinophils and basophils, both of which are prominent in allergic inflammation. The fact that any intervention with IL-5 should affect only these two cell types lessens the potential for significant impairment of host defences that would result from a pan-suppression of activated T cells, for instance. Thus, inhibition of IL-5, by reducing its systemic concentration, blocking its receptor association, or inhibiting downstream cell signaling, could provide a novel therapy for eosinophilic diseases. In animal studies, anti-IL-5 monoclonal antibodies produce a significant reduction in markers of allergic inflammation and are now being assessed in human clinical trials. There now follows a more detailed discussion of the role of IL-5 in eosinophilic diseases and an assessment of the various therapeutic targets for curtailing the effects of IL-5.

Eosinophils in inflammation

Eosinophils were observed in the spectrum of asthmatic patients some hundred years ago, but were initially recognized as a cell designed to combat parasitic infections. It is now well accepted that eosinophils are a major contributor to the tissue destruction that accompanies pulmonary inflammation and are the major cells infiltrating into the lung during asthma [1]. Eosinophils are produced from myeloid precursors in the bone marrow in response to a series of cytokines and are then released from the bone marrow and rapidly accumulate in tissue [2, 3] following the appropriate stimulus. In normal individuals there are very few eosinophils in the lungs but, in people with asthma, they accumulate dramatically [4]. When eosinophils are activated, they synthesize and release inflammatory cytokines, such as IL-5, and secrete a set of potent cytotoxic enzymes and proteins that can lyse epithelial tissues.

IL-5 is produced by various immune cells, including T cells, mast cells, and eosinophils. Allergic inflammation is associated with elevated expression of TH2 cytokines, both IL-4 and IL-5, in allergic diseases of the lungs, nose, skin, and gastrointestinal tract. In the lungs, the activation of $CD4^+$ T cells, and T cell expression of IL-4 and IL-5, has been demonstrated in bronchial biopsies and in bronchoalveolar lavage (BAL) fluid of both allergen-challenged atopic asthmatic and non-atopic asthmatic patients [5, 6]. Increased levels of IL-5 are seen in the BAL fluid of allergic subjects after segmental antigen challenge and correlate with levels of eosinophil-derived proteins [7].

The major source of IL-5 in allergic disorders is most likely the T cells [8,9], although numerous cells, including mast cells and eosinophils, can synthesize IL-5 [10–12]. Autocrine production of IL-5 by eosinophils may contribute to their own recruitment and activation, and to chronic eosinophil inflammation. Strategies aimed at inhibiting IL-5 biosynthesis in allergic disease need to consider the contributions of IL-5-producing cell types and the mechanisms involved in regulating IL-5 production in each cell type.

Studies of cytokine synthesis in the presence of cyclosporin A or protein synthesis inhibitors indicate that cytokines are independently regulated. Glucocorticoids are potent inhibitors of IL-4 and IL-5 secretions but are much less potent at inhibiting IFN secretion *in vitro* [13, 14]. The mechanism for this differential efficacy of steroids on various cytokines is unknown but would be particularly useful in asthma, reversing the exaggerated TH2 response [15, 16] including IL-5-induced eosinophilia.

Characterization and regulation of IL-5

The cDNA encoding murine IL-5 (mIL-5) was cloned in 1986 from a T cell line [17]. Using the mIL-5 cDNA as a probe, IL-5 cDNA was isolated from a human T cell leukemia line [18]. The amino acid sequence that was deduced from the DNA sequence for the mature IL-5 protein contains approximately 113 residues with several potential N terminal-linked glycosylation sites. There was little amino acid sequence homology with other cytokines. Upon expression, the human IL-5 cDNA induced IgM synthesis by stimulated human B cells. Using functional analysis, a correlation was observed between B cell activity and eosinophil differentiation activity, and it was established that a single cDNA clone encoded a protein that acted as a growth and differentiation factor for both B cells and eosinophils.

The regulation of IL-5 gene expression during T cell activation is controlled primarily at the level of transcription, although IL-5 mRNA is highly stable in activated mouse [19] and human T cells [19a]. Protein kinase C (PKC) activation is obligatory and sufficient for IL-5 mRNA induction [20] although IL-5 gene expression in response to PKC activation is enhanced by stimuli that increase intracellular calcium. Cyclosporin A, a cytokine inhibitor and immunosuppressant, inhibits the cytoplasmic to nuclear translocation of nuclear factor in activated T cells (NF-AT) by inhibiting the Ca^{+}-calmodulin-dependent phosphatase, calcineurin [21].

IL-5 induction by T cell receptor activation is sensitive to cyclosporin A [19]. However, like other cytokines, maximal IL-5 production requires signals contributed by the T cell receptor pathway as well as a costimulatory pathway provided by CD28 signaling [19, 22]. The CD28-mediated costimulation of IL-5 synthesis confers significant resistance to the inhibitory effects of cyclosporin A [22].

Although differences in the regulation of mast cell and T cell synthesis of IL-5 most likely exist, cyclosporin A has been found to block the induction of IL-5 mRNA in stimulated mouse mast cell lines [23]. It is important to note that cyclosporin A has been proven to be moderately beneficial in some individuals with severe asthma [24].

IL-5 gene expression in mouse and human T cells requires *de novo* protein synthesis [25]. This may distinguish signaling in T cells and mast cells because IL-5 mRNA was observed in anti-IgE stimulated human lung fragments in the absence of protein synthesis [26]. Studies of cytokine synthesis in the presence of cyclosporin A or protein synthesis inhibitors indicate that cytokines are independently regulated and the potential exists for the development of selective, small-molecule inhibitors of IL-5 synthesis for treatment of allergic disease.

IL-5 is found as a glycosylated homodimer of 45–60 kDa. Dimerization is required for full activity [25]. IL-5 interacts with a two-chain receptor comprising a unique transmembrane α subunit and a common transmembrane β subunit shared by IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors, thus creating a high- affinity receptor (Kd = 300 pM) [27]. The subunit is restricted to human eosinophils and basophils. Therefore, IL-5 exerts a limited function on human cells [28]. The IL-5 receptor, along with the IL-3 and GM-CSF receptors, mainly activates JAK2 kinases in response to ligand binding with subsequent activation of STAT5, a member of a family of transcriptional regulatory proteins.

Biological functions of IL-5

In addition to its effects on eosinophil generation, IL-5 can also have direct effects on the functions of eosinophils. IL-5 has been shown to have strong secretagogue effects on eosinophils. These effects are likely mediated via adhesion through the β_2 integrin as a prerequisite for degranulation [29]. IL-5 can also enhance eosinophil peroxidase levels *in vitro* in comparison to IL-3 and GM-CSF [30].

The direct linkage of IL-5 with eosinophils has been demonstrated through several laboratory experimental systems. IL-5 transgenic mice, for example, have a constant high number of eosinophils in their circulation [31]. On the other hand, mice deficient in IL-5 fail to elicit a pronounced eosinophilic response to aeroallergen challenge or parasitic infections. Although these mice can generate a basal level of functionally normal eosinophils, they appear to be insufficient to mount a pulmonary inflammatory response to aerosolized antigens and for normal host defense against parasites. These mice also have functional deficiencies in B cell functions [32]. Adoptive transfer of $CD4^+$ TH2 type T cells into the IL-5 deficient mice could, however, reconstitute an aeroallergen-induced blood and airway eosinophilia, lung damage, and airway hyperreactivity [33]. Additionally, it has been demonstrated

that ovalbumen- (OVA) specific T cell clones that produce IL-5 upon challenge can induce eosinophilic inflammation of the lung which can be completely suppressed by an anti-IL-5 antibody [34]. Further proof for the role of IL-5 in allergic disease models has been obtained through the use of neutralizing monoclonal antibodies. Anti-IL-5 monoclonals have been shown to suppress early phase nasal symptoms and late phase antigen induced eosinophilia in a murine model of nasal allergy [35]. Similar studies have established the strong correlation between eosinophilia and IL-5 and demonstrated that long-term treatment with an antibody does not have adverse effects on the immune system [36–38].

Evidence for the potential role of IL-5 in human asthma

Direct evidence for the role of IL-5 in airway hyperreactivity and eosinophilia was obtained in a placebo-controlled study of inhaled IL-5 in patients with allergic bronchial asthma [39]. Inhaled IL-5 increased airway responsiveness, infiltration of activated eosinophils in BAL fluids and showed elevated concentrations of eosinophilic cationic protein (ECP) in induced sputum.

Studies have also shown that the presence of IL-5 in induced sputum is a good indicator of eosinophilic inflammation in atopic and non-atopic asthmatics [40]. Increased IL-5 production by BAL cells has also been linked to an increased physiological response to allergen challenge [41]. In a controlled cross-over study to evaluate the effect and time course of repeated low-dose allergen challenge on airway hyperreactivity and airway inflammation, it was found that IL-5 production and eosinophilia were linked to hyperresponsiveness [42]. The levels of IL-5 induced by Japanese cedar pollen-induced allergic rhinitis were also linked to the episodic seasonal rhinitis in allergic patients [43]. Intriguing data has also emerged from systematic studies of patients undergoing specific immunotherapeutic regimens where immunotherapy converts TH2 responses to TH1 or TH0 responses and is associated with clinical improvement [44]. Similarly, patients that show a lower level of seasonal increase in IL-5 and IgE correspond to those responding better to immunotherapy [45].

Therapeutic potential for inhibition of IL-5

The mechanisms that underlie IL-5 production, release, receptor activation and cell signaling are all possible targets for therapeutic intervention. IL-5 biosynthesis is effectively inhibited by glucocorticosteroids [46, 14) and to a lesser degree by cyclosporin [47]. Therefore, it should be possible to identify other small-molecule inhibitors of IL-5 biosynthesis, particularly if IL-5 specific transcription factors or regulatory elements in the gene promoter can be identified. Similarly, biosynthesis

inhibitors of the expression of the α chain of the IL-5 receptor would also be a means of inhibiting IL-5 action.

Once released from the cell, IL-5 can be neutralized with anti-IL-5 monoclonal antibodies or soluble IL-5 receptors [48, 49]. This approach selectively inhibits the effects of IL-5, and in the case of anti-IL-5 antibodies, the action can have a very long duration [50]. The longevity of immunoglobins, which normally remain in the systemic circulation for many days, most likely explain their extended action. Monoclonal antibodies and soluble receptors that inhibit specific cytokines such as tumor necrosis factor (TNF), have had impressive therapeutic effects in the treatment of inflammatory diseases in clinical trials [51–53].

Cytokine receptor antagonists have been developed by mutagenesis, whereby the mutant protein binds to its receptor with high affinity but does not signal. An IL-4 receptor antagonist has been produced by this method [54] and this approach is reportedly successful for IL-5. While it should also be possible to find small-molecule, non-peptide antagonists, this has proved very difficult in practice, perhaps because the area of protein/protein interaction is too large for "small" molecules to be effective antagonists. Rather than targeting ligand-receptor interactions, the IL-5 signaling pathway is now becoming better elucidated and it may in the future be possible to find inhibitors of specific tyrosine kinases or transcription factors induced by IL-5.

Although there are a number of possible targets for inhibition of the IL-5 pathway, the most practical and specific therapeutic agent at the present time is neutralization of IL-5 by monoclonal antibodies. As discussed above, the IL-5 pathway is a relatively difficult one to approach for medicinal chemistry, but there are other areas of eosinophil biology, such as chemokines [55] and integrins [56] that are involved in chemotaxis and activation, which may offer more feasible targets.

Two major criteria will determine the use of an IL-5 inhibitor in the treatment of human disease or whether it has any role at all. The first is how critical IL-5 is in the pathogenesis of the particular disease or how effective an IL-5 inhibitor is in clinical trials. The second is how important IL-5 is in normal immune homeostasis and what effect its inhibition may have on host defenses. Practical considerations such as the severity of the disease, the availability and relative efficacy of treatment options, and the characteristics of the therapeutic regimen (e.g. oral or parenteral dosing, duration of effect) will also determine the value of an IL-5 inhibitor for a particular disease.

IL-5 production has clearly been demonstrated in asthma and is reduced after treatment or during remission. It is likely that in some, if not all, asthmatic patients [57] IL-5 plays a significant role in their disease and its inhibition will have a beneficial effect on symptoms and lung function. The most effective treatment for asthma are glucocorticosteroids, which suppress IL-5 biosynthesis *in vitro*. However, glucocorticosteriods also inhibit a range of other mechanisms, which may lead to undesirable side-effects over a long period of chronic use. In order to inhibit

eosinophil infiltration to the same extent as anti-IL-5 monoclonal antibodies, much larger doses of corticosteroids are required in animal models [58] than are used in treatment of human diseases. It is possible, therefore, that anti-IL-5 therapeutics, either alone or in combination with glucocorticosteroids, may be more effective in suppressing IL-5 action than present therapy, particularly in severe or steroid unresponsive asthma.

IL-5 inhibitors would be expected to be effective in some patients with allergic rhinitis and particularly in those with nasal polyps. In other diseases where IL-5 is thought to be involved, especially in those where relatively ineffective or no treatment is available, anti-IL-5 therapeutics could provide significant benefit and be rapidly adopted. Additionally, it may be possible in the future to identify those patients who are likely to respond to anti-IL-5 therapeutics by characterizing their gene expression profile.

It is possible that the inhibition of any element of the immune system may result in impairment of host defenses and this may occur if the activity of IL-5 is blocked. Although the effects of IL-5 inhibition on host defenses can only be properly assessed in clinical trials, there are a number of pieces of evidence that suggest that this could be of clinical significance. Raised IgE levels and eosinophilia are prominent features of parasitic infestation both in animals and humans. In mice, IL-4 and IL-5 production, respectively, have been shown to be increased [59, 60]. In the majority of studies, administration of an anti-IL-5 monoclonal antibody suppresses parasite-induced eosinophilia without having an effect on protective immunity [59–62].

IL-5 knockout mice have normal humoral and cytotoxic T cell responses, normal baseline levels of eosinophils and lack an eosinophilic response after worm infestation. However, these mice do not get an excessive worm load. In studies of parasitic infestation in IL-5 knockout mice and in the majority of studies on administration of anti-IL-5 monoclonal antibodies in normal mice, it is possible to completely abolish allergic responses without significantly impairing responses to parasites. Thus, it is reasonable to hope that clinical use of anti-IL-5 therapeutics should improve allergic inflammation without a major effect on host responses to parasites. However, it would seem sensible to limit the early clinical trials of anti-IL-5 medication to patients in areas where parasite infestation is uncommon.

In some murine models, there are data which suggest that local production of cytokines such as IL-5 and IL-4 may have anti-tumor effects under certain circumstances. However, their importance in tumor surveillance is not clear. For example, transfection of murine colon cancer cells with an IL-5 gene, and their transfer into mice, leads to increased eosinophil infiltration into the subsequent tumor and reduced tumor colony growth compared to unchanged tumor cells [63]. Neutralization of IL-5 by a monoclonal antibody only partially reversed this tumor inhibition. In contrast, IL-5 gene transfection of plasmacytoma or mammary tumor cells had no effect on tumor growth despite eosinophil infiltration of the tumors. In addi-