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Review

Chemokine receptors and their antagonists in allergic lung disease

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Abstract. The trafficking and homing of leukocytes in normal homeostasis and in disease is under the control of a variety of cytokine and lipid mediators. One family of small cytokines particularly involved in inflammation which has been identified is the chemokine family. Their action is mediated by a large superfamily of seven transmembrane spanning G-protein coupled receptors. One of the hopes in this field has been there may be selectivity in terms of which cells are recruited to sites of inflammation by virtue of their chemokine receptor expression pattern. This means that it may be possible to find antagonists of chemokine receptors that can selectively down regulate certain cell type recruitment, without provoking a generalized immunosuppression. In this review, we discuss the current state of understanding of the chemokine receptor field. The therapeutic potential of this field can be judged from recent data on the use of protein chemokine antagonists in allergic disease. The data so far obtained in animal studies point to the potential clinical uses of this emerging class of therapeutic agents.

Key words: Chemokines – Inflammation – Antagonists – Receptors – HIV

Chemokines and their receptors

Chemokines are a large family of small proteins that are involved in both, the basal leukocyte trafficking, and in the ac-

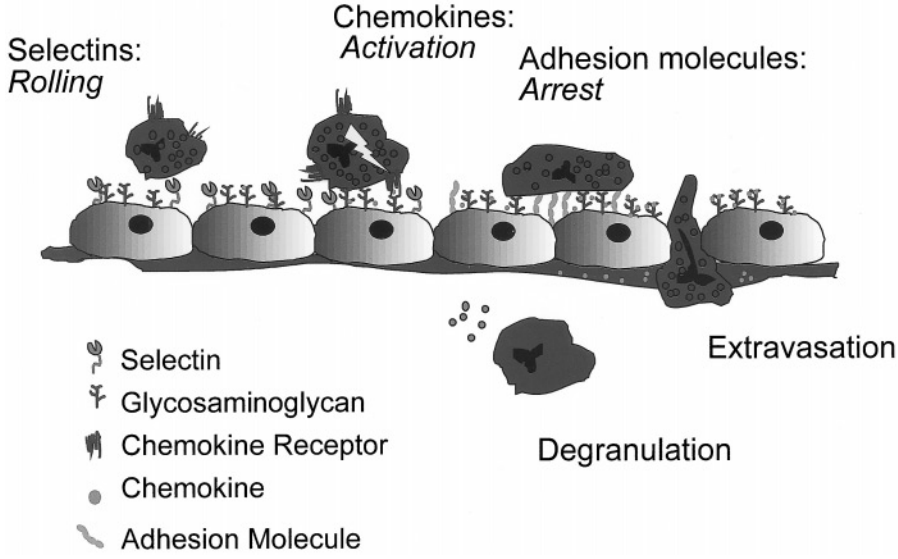

Fig. 1. Chemokine-induced transendothelial migration of leukocytes. Following activation via the selectin system circulating cells can roll along the endothelial surface, chemokines are proposed to form an immobilised or haptotactic gradient which directs the migration of cells towards the site of inflammation – and this gradient is stabilised by interactions with cell surface glycosaminoglycans. Finally, through a series of integrin-mediated events leukocytes can extravasate and then migrate through the tissues to their site of action.

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tivation and recruitment of specific cell populations during disease. The word chemokine comes from chemottractant cytokine, since these proteins were originally purified based on their ability to selectively induce the migration of specific cell types. As shown in Figure 1, the situation in vivo is complex, since chemokines have to play a role in the multistep process of arrest, rolling and transendothelial migration. Although there is only a limited amount of in vivo work supporting the hypothesis of the formation of haptotactic or immobilised gradients of chemokines which control cell migration, the model presented in Figure 1 is supported by a large amount of in vitro data on cells in culture.

Ten years ago, little was known about the factors which might act as the traffic controllers to regulate basal cell trafficking, as well as recruitment to sites of inflammation. The purification of some of these proteins by classical methods led initially to the identification of interleukin-8 (IL-8) [1] and subsequently monocyte chemotactic protein-1 (MCP-1) [2]. It turned out that despite a relatively low level of sequence identity at the amino acid level the three dimensional structures of these two proteins are almost superimposable [3, 4]. The overall fold of the monomers is conserved for all the chemokines, in fact this has become the mechanism by which several new chemokines have been identified, even before their biological activity has been completely confirmed. To illustrate this, we have overlaid the three-dimensional structures of interleukin-8, MCP-1 and fractalkine (the only member of a third class of chemokine) as a ribbon diagram in Figure 2. There are many differences in the quaternary structure of chemokines, but the role played by this quaternary structure is still relatively controversial – since these complexes or multimers tend to form in solution only at high concentrations, which many groups have claimed are physiologically irrelevant. One hypothesis is that cell surface glycosaminoglycans enhance this oligomerisation process. This is discussed later in this review.

The sequencing of the amino terminal ends of chemokines showed different motifs of cysteine residues. In IL-8, the residues are separated by a single amino acid to form a CXC motif, whereas in MCP-1 the residues are adjacent, and form a CC motif. This allowed the division of chemokines into two main subclasses depending on this spacing pattern: the CXC (or α) subclass and the CC (or β) subclass. Recently, two new motifs have been identified: the C chemokine, lymphotactin [5], and the CX_3C chemokine, named either fractalkine [6] or neurotactin [7] by the groups who identified it. For a long time it was thought that the two main subclasses had separate activities, since the CXC subclass principally activates neutrophils whilst the CC-subclass activates the other leukocyte types such as T cells, eosinophils, monocytes/macrophages, basophils and dendritic cells. Thus the CXC chemokines were thought to be associated with

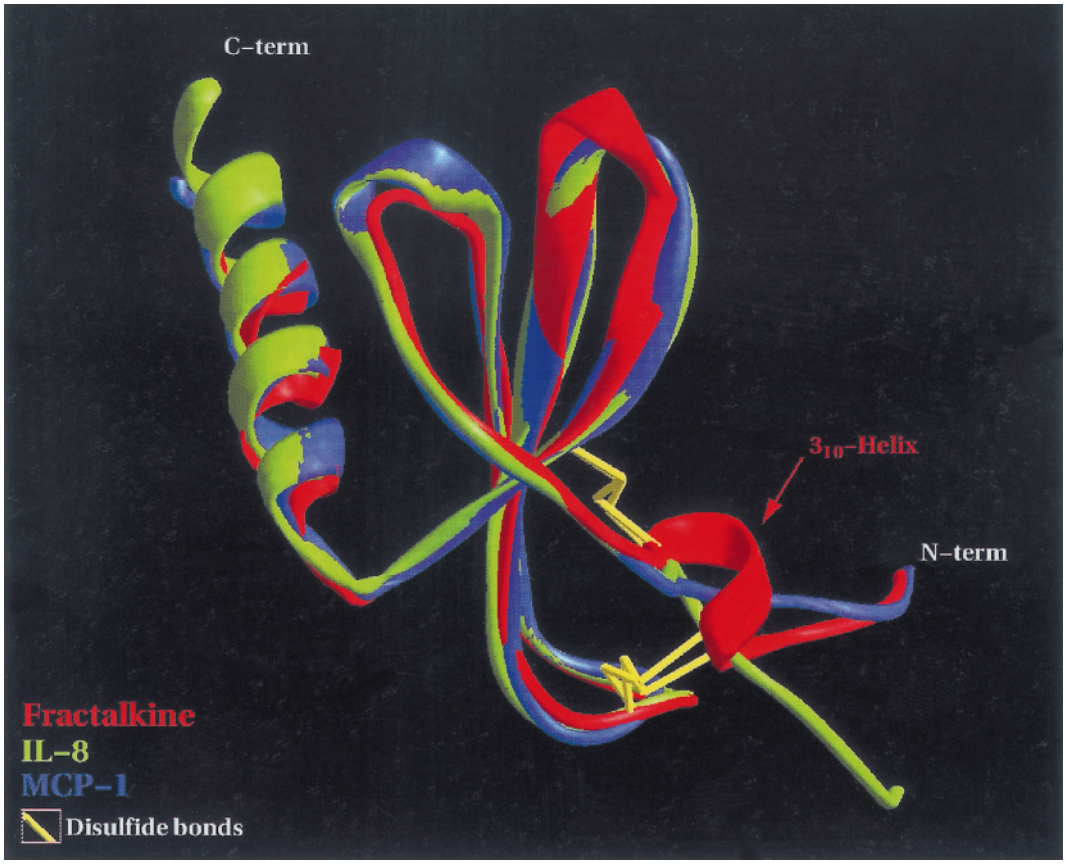

Fig. 2. Comparison of the three-dimensional structures of human IL-8 (green), MCP-1 (mauve) and Fractalkine (red) (taken from the sequence of the EST Z44443). The IL-8 structure is taken from the PDB entry 1-IL-8. The model for the chemokine domain of fractalkine was built using the Swiss-model server [9].

acute inflammation, characteristically accompanied by plasma fluid exudates and neutrophil accumulation. Chronic inflammation is characterised by a dense cellular infiltrate comprised of lymphocytes, eosinophils, or monocytes/macrophages and is thus associated with the CC chemokines. However, as studies on chemokine biology have been elaborated, this rule has clearly broken down – for example, the identification of the CXC chemokine receptor CXCR3 highly expressed on activated T cells [8], suggests a potentially important role for CXC chemokines in chronic inflammation.

The chemokine family has exploded in complexity over the last few years. In the early years, new members of the family were purified from cell culture media by classical protein chemistry. Later, standard cDNA cloning techniques were used. More recently there has been a dramatic increase in the number of new chemokines, caused by the identification of chemokine-like sequences in expressed sequence tag collections. These are large numbers of DNA sequences obtained by sequencing clones at random from a cDNA library. The availability of this data over the World Wide Web, combined with the fact that chemokine open reading frames are typically around 100 amino acids, and ESTs are typically 300–500 bases long, has made them relatively easy to find [9]. The number of new chemokines has become overwhelming for comparison purposes. The simplest representation is shown in Figure 3 where the chemokines are clustered in a dendrogram, where chemokines with the most similar sequences being closest. Even so, it is clear biologically, that sequence similarity is not an accurate reflection of functional similarity. As will be discussed later, small modifications to sequence can have a dramatic effect on the activity of the protein.

The availability of many more new ligands over the past four years has meant that a number of new receptors have now been formally identified. Many cDNAs which encode molecules similar to chemokine receptors were cloned during the early 1990s using reverse transcriptase-PCR strategies, with degenerate primers. The challenge over the past few years has been to identify the correct ligand for each receptor. The pharmacology of the receptors has been reviewed extensively elsewhere [10] – but at the time of writing there are published reports of five human CXC chemokine receptors, nine CC chemokine receptors, CX_3C chemokine receptor, and the C chemokine receptor, Duffy antigen, and various murine and virally encoded chemokine receptors, (the data are summarised in Table 1).

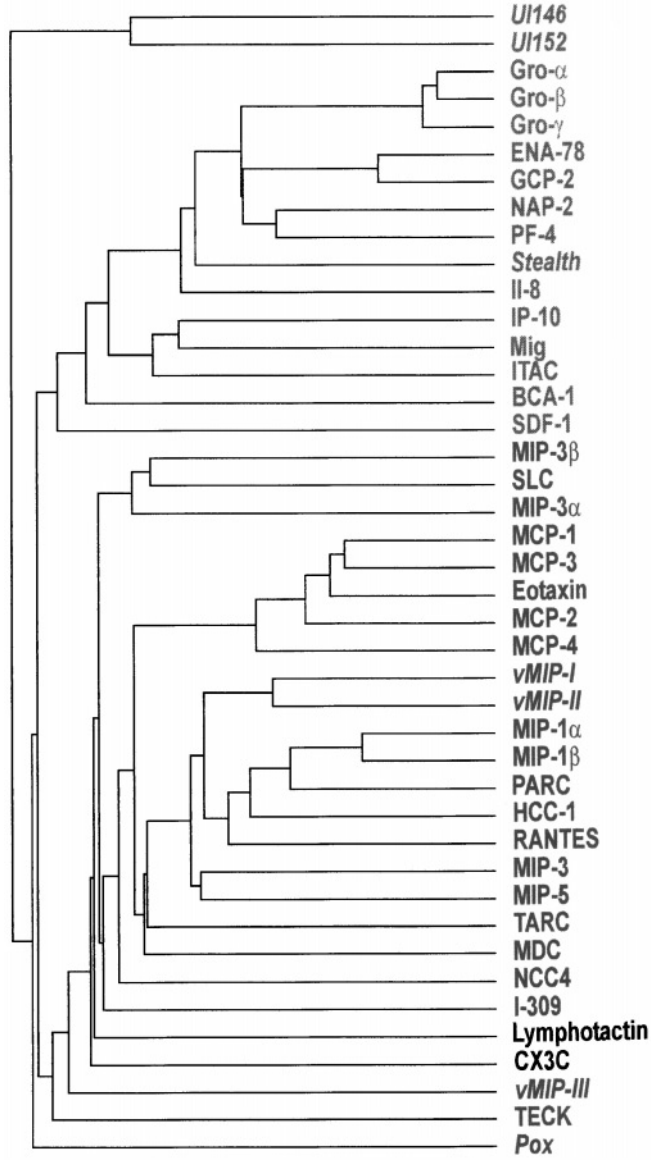

Fig. 3. Dendrogram showing the similarities between the human, virally encoded chemokine protein sequences. The sequences cluster in terms of the level of identity pairs of amino acids. The further to the right that lines branch in on the diagram, the higher level of sequence identity the two proteins have.

The problem of selectivity

One of the initial hopes in this field was that chemokine receptor antagonists would be useful therapeutic agents in controlling inflammatory disease. To do this, the antagonists must selectively control the activation and recruitment of individual groups of leukocytes – and therefore offer a distinct advantage over currently available therapies, which tend to be non-selective. As more and more receptors have been identified, then the number of receptors reported to be used by each chemokine has also increased. This has led to the suggestion that there is simply too much redundancy in the chemokine network for selective therapeutic intervention. That is to say, that in the majority of cases, since any one chemokine can bind several receptors, and most receptors bind several chemokines, the disruption of any particular ligand/receptor interaction might be expected to have little effect on a given inflammatory disease state.

At this stage, this is still an overly pessimistic viewpoint for a number of reasons. First, most of the ligand binding data that is discussed is obtained from in vitro experiments, where receptors are expressed at very high levels on the surface, often 10–100 times higher than is actually seen in primary cell types. Little data is available as to how this overexpression effects selectivity. Second, the cell context may well be important – since it defines which G-proteins are present, and

Table 1. The human chemokine receptors and their ligands. The cell type expression refers to the most important cell types, although it should be stressed that many exceptions to this simplified account have been reported. As discussed in the text, it should also be borne in mind that the expression level of a particular receptor on a given cell type will depend on the activation step and priming state of that cell type. In addition, considerable variation in expression level has been seen from individual to individual, further complicating the picture (taken from ref. 10).

Receptor	Ligands	Main cell type	Accession code	Accession code murine homologue
CXCR1	IL-8	neutrophils	P25024	
CXCR2	IL-8, NAP-2, Groα, ENA-78	neutrophils	P25025	P35343
CXCR3	IP-10, MIG, ITAC	activated T cells (T_H1)	P49682	
CXCR4	SDF-1	naïve T cells, B cells	P30991	P70658
CXCR5	BCA-1	B cells	X68149	
CCR1	MCP-3, RANTES, MIP-1α	activated T cells, monocytes, eosinophils, DCs	P32446	P51675
CCR2	MCP-1,-2,-3,-4,-5	monocytes, macrophages, activated T cells	P41597	P51683
CCR3	eotaxin, MCP-3,-4; RANTES	eosinophils; basophils, activated T cells (T_H2)	P51677	P51676
CCR4	TARC, MIP-1α RANTES, MDC	activated T cells (T_H2); basophils; platelets	P51679	P51680
CCR5	MIP-1β, RANTES, MIP-1α	activated T cells, monocyte/macrophages; DCs	P51681	P51682
CCR6	MIP-3α	DCs, T cells	P51684	
CCR7	MIP-3β	B cells, T cells, DCs	P32248	P47774
CCR8	I-309	monocytes; macrophages;	P51685	P56484
CCR9	CC chemokines	non-haematopoietic cells	Y12815	
Duffy antigen	IL-8, Groα, RANTES, MCP-1	erythrocytes	Q16300	
CX_3CR1	fractalkine (neurotactin)	NK cells; CD8 T cells	P49238	

they in turn may alter the conformation and activity of the receptor. Different cell backgrounds have in fact given conflicting results, as is well demonstrated by the ligand binding profile of one of the CC receptors, CCR4. When this receptor was originally cloned in our laboratory and expressed in oocytes, it was clearly shown to be activated by RANTES, MIP-1α and MCP-1 [11]. RANTES and MIP-1α were shown to bind to CCR4 when expressed in HL60 cells [12]. Following this publication, other laboratories have shown that CCR4 is the receptor for the more recently described chemokines TARC [13] and MDC. The key to understanding such conflicting results is that the level of receptor expression and correct G-protein expression may determine the pharmacology of a given receptor. The level of functional receptors varies dramatically between different cell systems. Many ligands may give a response in a calcium flux assay at high receptor density, but these results may be less significant at physiological receptor levels. In any case, it is essential that calcium flux studies identifying new ligands for chemokine receptors are backed up as early as possible with receptor binding studies on more than one cell type. Third, although a seven transmembrane receptor may bind several ligands with equal potency in equilibrium binding assays, there may still be one ligand which is capable of competing away all the others, and thus dominating the physiology of the system. This breakdown of cross competition is counter-intuitive. It implies that the receptor-ligand binding is not at equilibrium, but is in a steady state. The limitations of cross-competition studies have been very elegantly shown in the neurokinin receptor system [14, 15]. The receptor clearly exists in a number of conformations, and one explanation of the lack of cross-competition requires a slow conformational change of the receptor ligand complex, which is slower than ligand association/dissociation. Data showing breakdown of cross-competition has been reported in the chemokine area [48], but since there are relatively few groups doing all of the homologous and heterologous competition experiments required to support such a hypothesis, the data in this area are still relatively limited.

Although in vitro the receptor and ligand may bind and cause a cellular response, in vivo they have to be expressed in the same place and at the same time. Thus it is clear that the control of both the ligand and receptor expression is extremely important, and at least for the receptors this question has largely been neglected. However, the pioneering work of Loetscher et al. which showed that CCR1 and CCR2 were upregulated in the presence of IL-2, clearly demonstrated the importance that cytokines play in the chemokine system [16]. Another example is that the pro-inflammatory cytokine IFNγ, upregulates the CC chemokine receptors CCR1 and CCR3 on neutrophils, a cell type previously thought to only be activated by CXC chemokines [17]. The role of presentation by GAGs is not well defined in vivo in how they alter the pharmacokinetics of the formation and destruction of chemokine gradients, but these are essential questions and are the subject of much active research.

Finally, to be therapeutically useful, chemokine antagonists need to be potent, and selective in the sense that they do not bind to other distantly related seven transmembrane receptors. However, it is far from clear what the optimal receptor binding profile for a chemokine therapeutic in any given disease area is, and data from animal experiments discussed below shows that it may be important to block more than one chemokine receptor in any disease process.

Chemokines and their receptors in asthma

Allergen provocation of allergic asthmatics has long been known to result in two phases of bronchoconstriction in the majority of patients. The early phase of bronchoconstriction is the result of cross-linking of IgE to its receptor and the release of mast cell granule products into the airway. The late phase reaction, taking place some hours later is associated

with a cellular influx, containing principally eosinophils and T-cells. It is clear that in the clinical setting there is a strong correlation between the numbers of eosinophils and eosinophil products detected in the bronchoalveolar lavage (BAL) and the extent to which lung function is impaired [18, 19]. Eosinophil granules contain many products including peroxidases and the major basic protein which have been detected in the lavage of asthmatics, which have been shown in a variety of experimental settings to be potential agents of damage for such patients. It has been suggested that the continual inflammation of the lung, by repeated influx and degranulation of eosinophils and other leukocytes, leads to the long-term changes resulting in an increase in broncho-hyperreactivity, and also to successive structural changes such as fibrosis. In addition, in fatal asthma, concentric rings of activated eosinophils can be seen embedded in layers of mucus and this gives a very vivid picture of the successive waves of cellular recruitment which lead to total blockage of the airways and ultimately to death [20].

The early identification of eotaxin [21] as a major primary eosinophil recruiting factor in models of allergic lung inflammation in the guinea pig, led rapidly to the identification of its human homologue, and from that the receptor, subsequently named CCR3. CCR3 has been shown to be the principal chemokine receptor on most human eosinophils and given the importance of this receptor/ligand pair, has been the focus of much attention to find small molecule inhibitors. In addition to eotaxin, interleukin-5 (IL-5), the eosinophil differentiation and priming factor, clearly plays an important role in the production and release into circulation of eosinophils, and the synergy between IL-5 and eotaxin in coordinating inflammation has been convincingly demonstrated in guinea pig lung models [22]. The other cell types which are important in asthma are the epithelium, the dendritic cell, alveolar macrophages and the T-cell. The details of chemokine expression by the epithelium are complex, but in studies of ovalbumin challenged mice, there is a clear upregulation of the receptor CCR3 on epithelial cells (A. J. Coyle et al., unpublished observation). This correlates well with human studies, where there is evidence that the epithelium is also a major source of chemokines, such as eotaxin and MCP-4. The dendritic cell is the professional antigen presentation cell, and therefore has long been supposed to play a role in the development of the immune response in allergic asthma. The identification of human CCR6 [23–25] and subsequently human CCR7 as dendritic cell chemokine receptors, with specific ligands, show that they may have a role to play in the development of the allergic response. The relatively tight receptor-ligand selectivity offers promise for selective intervention by modulating dendritic cell function. The debate is what physiological consequences of such an intervention would be. The fact that these receptors have been only recently identified, means that studies of their role in the development of allergic models of lung inflammation are still in their infancy. Macrophages are present in the lungs during the inflammatory response. In allergen provocation studies on mice both CCR2 and CCR5 are upregulated on the alveolar macrophages, as revealed by staining with Mac-1 (A. J. Coyle et al., unpublished observation). Again, this is suggestive of a role in the development of the lung inflammatory response, and it will be interesting to see whether a similar upregulation is seen in asthmatics. The role of the chemokine system in the T-cell response has been further elaborated by the identification of different expression patterns of chemokine receptors on T-cell populations, as discussed below.

The balance between T_H1 and T_H2 in inflammatory and autoimmune diseases

Diseases involving the immune system can be classified as being either T_H1 or T_H2 type-depending on the spectrum of cytokines produced by activated T helper (T_H) cells. T_H1 cells produce mainly IL-2, IFN-γ and IL-12 whereas T_H2 cells secrete IL-4, IL-5, IL-10 and IL-13 [26]. This is a simplification, since the understanding of the T_H1/T_H2 system is much better developed for the mouse. However, it serves as a useful model when discussing chemokine and chemokine receptor profiles in auto-immune disease from allergic inflammation. From a disease perspective, T_H1 cells are the major players in the inflammation characterized by activated T-cells and macrophages, and have been associated with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and also in delayed type hypersensitivity reactions. T_H2 cells are involved in the production of IL-4, and IL-5, and in the responses leading to eosinophil and basophil recruitment. They are therefore implicated in the pathogenesis of allergic inflammatory diseases such as asthma and atopic dermititis [27]. The selective recruitment of different leukocyte subsets is necessary for efficient regulation of the immune response. The importance of chemokines and chemokine receptors controlling the recruitment of T-cell subsets was demonstrated by the activity of RANTES to attract CD4 memory rather than naive T-cells [28]. More recent results demonstrate that chemokine receptors are differentially expressed on naive cells, as well as T_H1 and T_H2 subsets and that this expression can be modulated by cytokines. On naive T cells, CXCR4 is the major chemokine receptor expressed, a finding which is consistent with the proposed role of its ligand SDF-1, in directing basal lymphocyte trafficking. On activated T-cells, CCR3+ cells are predominantly of the T_H2 type [29]. These findings were extended to show that T_H2 cells also express CCR4 whereas T_H1 cells express CXCR3 and CCR5 [17]. Chemokine receptor expression on T lymphocytes, together with tissue-specific chemokine expression, are therefore important factors in controlling the composition of lymphocyte infiltrates in different types of inflammatory pathology. Selective recruitment may be as important as differentiation in controlling whether a T_H1 or T_H2 type response is produced in a disease situation.

Antagonising chemokine function can alter the course of inflammatory disease

When RANTES is expressed in the bacterial host *E. coli*, the initiating methionine is retained, resulting in Met-RANTES. This protein can block functional responses of cells to RANTES and MIP-α in both chemotaxis and cell calcium studies in vitro [30]. This effect is selective for RANTES receptors,

and the protein has no effect on IL-8 or MCP-1-induced cell responses. In most primary cell systems, Met-RANTES acts as an antagonist of the RANTES-induced response. In some recombinant receptor expressing cell lines with very high expression levels, Met-RANTES can induce a functional calcium response. Strictly speaking therefore, we should describe Met-RANTES as a weak partial agonist, and this weak agonism is cell type-dependent. In vitro Met-RANTES will prevent the recruitment and activation of a variety of cell types, including T-cells, monocytes and eosinophils. In vivo, the molecule is also active. Intraperitoneal Met-RANTES treatment in the collagen induced murine model of arthritis causes delay in the disease onset, and a reduction of severity and the number of affected paws. This protective effect is only achieved if the antagonist is administered prior to the onset of the arthritic condition [31]. In a model of another autoimmune inflammatory condition, glomerulonephritis (in which there is also an associated macrophage accumulation), Met-RANTES is highly effective. A comparative study using the functional antagonist Met-RANTES and a monoclonal anti-MCP-1 antibody [32], showed that whilst Met-RANTES caused a significant decrease in macrophage and T-cell infiltration, it had no effect on the fibrosis.

We have also shown that administration of Met-RANTES can alter the course of a murine model of allergic airways inflammation. Repeated sensitisation with ovalbumin causes the recruitment of $CD4^+$ T-cells and eosinophils, and a subsequent increase in the bronchial hyperreactivity. Treatment with Met-RANTES significantly reduces the accumulation of T-cells and eosinophils in the airways. This is achieved with low doses of protein (0.04 mg/kg), which inhibit 95% of the eosinophil accumulation into the bronchoalveolar lavage fluid. Peroxidase staining confirms that this reduction of accumulation of cells into the BAL is also mirrored by a reduction of the number of cells in the interstitial spaces. Similarly, the accumulation of T cells is reduced by 80–90% compared with control animals. Even more striking though is the inhibition of bronchial hyperreactivity. The sensitised untreated animals show a marked hyperactivity when challenged with methacholine, as is the case in human asthmatic patients. Met-RANTES treated animals showed a significant reduction in hyperreactivity. Mucus production is also blocked, as can be seen by both a reduction in mucus and also by the staining of the goblet cells in the bronchial epithelium. Given the prevalence of airways which are completely blocked by mucus in fatal asthma, this result is highly significant. Whether or not this is due to a direct effect of Met-RANTES on the goblet cells themselves, or an indirect effect, perhaps through mast cells is not clear at this stage. The latter hypothesis is supported by the observation that in models of murine footpad swelling, Met-RANTES exerts its effect by preventing degranulation of subdermal mast cells. These data are supported by a second study comparing two approaches: receptor blockade using Met-RANTES, and different anti-chemokine antibodies. The results in terms of cell recruitment at day 21 are shown in Table 2. Here we can see that for recruitment to the lavage fluid, the Met-RANTES effects are even more potent than seen for antibodies raised against eotaxin, the specific CCR3 chemokine. Taken together with the histological studies in the mouse model of lung inflammation in this second study, we are confident that the effects on cell recruitment to the BAL reflects tissue distribution. A word of caution is due here, since the study does not include complete dose response curves [33], and we have assumed that the dose used ensures a maximal response. However, the results strongly suggest two things. First, that receptor blockade is more effective than addition of an antibody against one particular chemokine at reducing lung inflammation. This underlines the fact that inflammation is an orchestrated response of a number of mediators, and therefore interfering with an individual ligand is unlikely to be effective. Second, blockade of multiple receptors is a possible strategy for therapy in both the skin [34] and the lung [33]. At this stage, we do not know which of the receptors effected by Met-RANTES are essential for the anti-inflammatory properties, but the receptor profile used by Met-RANTES gives us a starting point in our search for useful small molecules.

These results lend support to the notion that despite the apparent redundancy in the chemokine family receptor antagonism can significantly reduce inflammation. However, there still remains a considerable amount of work to precisely identify which receptors are upregulated, and on which cell type in disease such as allergic asthma. In addition, we understand more about the effect that existing therapies (such as the use of β-adrenoreceptor agonists and corticosteroids) will have on the expression of key chemokines and their receptors.

Searching for small molecules – the classical mechanism of receptor blockade

Modified chemokines, and antibodies against receptors are an important part of our tools to be used in understanding the role of chemokines in disease. From a therapeutic standpoint,

Table 2. OVA-induced leukocyte infiltration in the airways after human chemokine blockage. Percentage reduction of the number of leukocytes in BAL of OVA treated mice after blockade of the chemokine network with either antibodies or Met-RANTES. Bronchoalveolar lavage fluid (BAL) was obtained 3 h after OVA treatment on day 21. Values are given as percentages relative to the mice treated with ovalbumin and a control antibody, which are taken to be 100%, (data taken from ref. 33).

	Macrophages	Monocytes	T-cells	Eosinophils
Control (total numbers)	$(122 \pm 12) \times 10^3$	$(25 \pm 3) \times 10^3$	$(130 \pm 22) \times 10^3$	$(870 \pm 92) \times 10^3$
Met-RANTES	−17 ± 1%	−44 ± 8%	−95 ± 4%	−91 ± 2%
Anti Eotaxin Ab	−17 ± 4%	− 7 ± 1%	−21 ± 4%	−60 ± 7%
Anti MIP1α Ab	− 2 ± 0.3%	0	0	−23 ± 2%
Anti MCP-1 Ab	−58 ± 3%	−53 ± 5%	−89 ± 6%	−78 ± 12%

they are very useful – since they can be rapidly progressed through to the clinic, where pivotal "proof of principle in man" studies can take place. This means testing whether blockade of the chemokine system has any real benefit when we look at real disease in the human context rather than simply animal models. It would serve us little, to be able to show that chemokine receptor antagonists can block inflammation in animal models, only to find out in the clinic that such inflammation and recruitment are not the fundamental cause of the bronchial hyperactivity seen in man. However, proteins have some limitations as therapeutics, including cost, and route of delivery (they cannot be administered orally). The search in most pharmaceutical organizations has therefore been to find a small molecule, which would selectively block chemokine receptors, and therefore give similar effects to those already seen in animal models from studies with modified chemokines or antibodies.

This search for small molecule antagonists of chemokine receptors has been a highly active area of pharmaceutical research in the last few years. The approaches taken by many groups are similar – using solid phase assays with recombinant receptors, radiolabelled chemokines and automated assay systems [35]. Using receptors present in membranes from cells which express functional chemokine receptors at high levels it is possible to screen some 10 000 molecules per day to look for inhibition of binding. There has been a lot of debate about the type of molecules that should be screened in this manner. Early screening experiments used compounds made for other projects and which had been shown to be inactive in other assays – a random approach. This is only possible in organizations which have relatively large collections of compounds, and so many groups have made large collections of molecules rapidly using combinatorial chemistry, with the emphasis on quantity rather than quality. Even in large companies, this approach may prove to be self-defeating, since radioligand binding assays typically cost \$1–2 per sample, which limits the number of compounds which can be screened purely on economic grounds. More productive approaches have used focused sets of molecules that can be made based on templates, or chemical themes, which are known to occur frequently in small molecules which are active against seven transmembrane receptors – for example benzodiazepines or diphenylmethyl groups. Since small molecules interacting with seven transmembrane spanning G-protein coupled receptors have been an extremely fertile source of therapeutically useful molecules for the pharmaceutical industry, many such libraries exist. (Examples of other compounds acting on receptors important in the respiratory field include anti-histamines, β-agonists, muscarinics, luekotriene receptors etc.). This approach has been far more successful.

Within the cytokine world there has been much debate as to whether small molecule antagonists of cytokine receptors would ever be found [36]. For cytokines, such as Interleukins 1, 2 and 5 large high throughput screening campaigns were run on the early 1990's by a number of different companies using several different formats, but with disappointingly few results, none of which have so far been reported to have clear activity in cell based assays. The best explanation for this failure is that when a cytokine and a receptor bind, the interaction surface between the two proteins is relatively large [37]. Unfortunately, the molecules screened typically have a molecular weight of less than 500 (which is generally a requirement if the molecule is to be orally available). Such small molecules are unlikely to be able to cover all of the key interactions in the receptor/ligand binding surface. Under these circumstances, success or failure will depend on just how localized the key residues in the receptor involved in ligand binding really are. Recent progress in search for cytokine antagonists and agonists supports this analysis. Dimeric peptides which can agonize and antagonize cytokine receptors have been found with nanomolar potency, but relatively high molecular weight (>2000) [38, 39]. More recently small molecule mimetics of granulocyte colony stimulating factor (G-CSF) have been described but although this is a major breakthrough, the molecules are still only of micromolar potency [40].

With this chequered history in mind (the optimism of the seven transmembrane spanning receptor family combined with the pessimism of the cytokine receptor family), there was much excitement when the first real small molecule inhibitors of the IL-8 receptor, CXCR2 were reported [41], the patents had appeared earlier, 42]. SB 225002 is a urea-based inhibitor of CXCR2, which has nanomolar potency, and also a high degree of selectivity over CXCR1. It is selectively able to block neutrophil migration in rabbit models of inflammation, suggesting that CXCR2 plays a key role in this process. These data are somewhat at odds with previous data in the rabbit, which suggest CXCR1 as the key receptor [43] – however, it does serve to highlight how useful selective small molecule antagonists are going to be in dissecting out the molecular mechanisms of many inflammatory diseases. The role activity of this compound in the lung injury process has so far not been described. The interest in CXCR4 as the co-receptor for HIV, has focussed several groups on the task of trying to find a small molecule inhibitor. There are three reports of antagonists of CXCR4 – AMD3100, a bicyclam [44, 45]; and two peptides [46, 47].

Published data on the antagonists of CC chemokine receptors are relatively rare. Hesselgesser et al. [49], have reported a series of 4-hydroxypyridine derivatives of the classical diphenylmethyl scaffold. These compounds are able to block signaling via calcium flux in the sub-micromolar concentration range. Given the interest of the authors in autoimmune disease, the work has focussed on CCR1 – and the compounds are selective against CCR5. No data for the other receptor known to be important in T_H1 response (CXCR3) are given, and in addition there are unfortunately no data for the T_H2 linked receptors CCR3 and CCR4. Similar compounds have been described in the patent [see 42 for review]. The compounds are based on diphenylmethyl scaffolds, a motif found in many molecules binding tightly to seven transmembrane receptors. It is also important to point out that there is no clear consensus on what receptor binding profile is needed for a molecule to be potentially useful in lung inflammation. To repeat, although CCR3 is clearly the key receptor on eosinophils which have long been suspected to be the key cell in human asthma, the relative contribution of T_H2 type T-cells is still not defined. The work on modified chemokines suggests that being able to selectively bind more than one type of chemokine receptor may be an advantage.

Glycosaminoglycans – blocking the presentation of chemokines

The large number of chemokines, and receptors, and the way in which an individual receptor binds many overlapping chemokines raises the question of whether there is too much redundancy in the chemokine system. From a drug design perspective this question is critical – since if the consequence of one receptor being blocked is that another receptor type can take over the same role, then chemokine receptors would cease to be interesting therapeutic targets. However, there are several levels of control in the body, which result in a greater degree of specificity, several of which have been addressed in this review: oligomerization on cell surfaces may be one of these. It is a well-known fact that chemokines bind to glycosoaminoglycans (GAGs) [50, 51], a property that has been used extensively in their purification on heparin sepharose columns. This property of binding to GAGs would certainly be essential for their immobilization on endothelial cells lining the vessel walls. This is one place where they create the haptotactic gradient in order to fulfill their principal role, directing cellular trafficking of leukocytes. The binding of chemokines to heparin induces oligomerization [52]. The K_D for this oligomerization ranged from 1–25 nM, for the 4 chemokines tested, RANTES, MIP-1α, MCP- 1 and IL-8, indicating a sensitive mechanism of creating elevated localized concentrations. Furthermore, it has been shown that chemokines display a selectivity for different GAGs [51], and that certain chemokines such as RANTES show two orders of magnitude difference in their K_D values for GAGs such as heparin compared with chondroitin sulfate (G.S. Kuschert et al., unpublished observation). This selectivity in the binding of chemokines to different types of GAGs, and the different expression patterns of cell surface in disease, could thus introduce a level of control whereby oligomerization on GAGs could specifically favor an elevated local concentration of a certain chemokine. In inflammatory disorders, the cell surface expression of GAGs has been shown to be modified. An example is the increased cell surface chondroitin sulfate on atherosclerotic plaque tissues [53, 54]. Thus, whilst in solution, where the dissociation constants for ligand dimerization are several log units above the concentrations required for biological activity and receptor binding [55, 56], the dissociation constants on the surface of cells may well be in the physiological range. This opens up another possible way of getting small molecules to interfere with the chemokine system. If a small molecule can be made which interferes with the binding of chemokines to glycosaminoglycans, then it would be possible to disrupt the immobilized gradient of oligomerized chemokine, and therefore prevent the directed migration of the inflammatory cells. Although no such molecules have been described so far, two further lines of evidence support the concept. First, the observation that among its many activities in the clinic, low molecular weight heparin is indeed used as an anti-inflammatory and has been shown to block leukocyte recruitment in vivo. Second, we have shown that molecules as small as disaccharides can interfere with the binding of chemokines to GAGs, and that this binding is not absolutely dependent on the number of charged interactions [57].

Viral approaches to subverting the inflammatory response

In their attempts to evade the host immune system, and create a better environment for themselves, mammalian viruses have evolved a wide range of strategies. These include the expression of many cytokines, chemokines and chemokine receptors (recently reviewed in Wells and Schwartz [58]. Understanding the mode of action of these virally encoded cytokines will clearly enhance our understanding of immunology, and may lead to ideas which enhance our ability to design therapeutic molecules. The low level of sequence conservation between the viral and human proteins may mean that they will be immunogenic. However, it is possible that these proteins may also have evolved to escape recognition by the host system. Viral chemokine receptors were first identified in human cytomegalovirus – where the US28 open reading frame was shown to be a functional receptor for CC chemokines but not CXC chemokines, such as IL-8 [59]. Links with the expression of the viral receptor in diseases such as atherosclerosis [60] remain speculative, although attractive hypotheses. Viruses have often been suggested to be at the root of many chronic inflammatory diseases, but this is very difficult to prove formally.

The most intensely studied viral chemokines to date are those from herpesvirus 8 [61–63] which encodes three chemokines. These molecules show the highest protein sequence similarity to human MIP-1α. Interestingly, the vMIP-II has been shown to be relatively promiscuous, and is the first natural chemokine to bind to both CC and CXC chemokine receptors. The protein is able to block infection of cells by HIV, but is most potent when CCR3 is being used as the co-receptor. Its broad selectivity raised hopes for its use as an antiviral, but its potency in viral infectivity assays has been somewhat disappointing. In some assays, such as monocyte migration, vMIP-II has been shown to be a partial agonist [63], and on eosinophils appears to be a full agonist [62]. So far, none of these molecules have been tested in in vivo models of lung inflammation, but based on the results seen so far, these studies will certainly further develop our understanding of chemokine networks.

One final twist in the viral chemokine story is the identification of a soluble protein capable of binding chemokines with picomolar affinity, termed p35 protein [64]. This is presumably involved in antagonizing the activity of chemokines during viral infection and, again would be predicted to have anti-inflammatory actions in many in vivo models. In addition, the three-dimensional structure of the chemokine/p35 complex will offer insights into how small molecules that bind to chemokines and inactivate them, may be designed. If the virus uses both chemokine binding and chemokine mimetic strategies to evade and modulate the immune system, this indicates that small molecule pharmaceuticals that attempt either of these strategies may well have therapeutic utility.

Conclusions

Recent advances in the number of chemokines and their receptors identified means that we now have a much clearer picture of the players which could be involved in the inflam-

matory and immune components of diseases such as asthma. What is required now is to understand the role of these proteins in the pathology of disease. The first step in this process has been understanding the cell biology of the key cells (eosinophils, T-cells, macrophages, mast and dendritic cells) from a chemokine perspective. The second step is to correlate the data obtained from animal models of lung inflammation largely available for the mouse to the human pathology. The third step is proof of concept in the real human disease, and these experiments are much more complex. Although many groups have described antibodies to chemokine receptors, so far no clinical data have been presented. In any case, given the interest of pharmaceutical companies in this area, it would be reasonable to assume that such studies would be rapidly followed up with small, orally bioavailable molecules. From the data obtained so far, the idea that chemokine receptor blockade will be a useful adjunct to current therapy for inflammatory lung diseases is a promising one. It remains to be seen whether the first clinical studies will bear out this promise.

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