Antimicrobial and related activities of chemokines

Osamu Yoshie

Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan

Introduction

Chemokines are now known to play pivotal roles in both innate and acquired immunity primarily through their chemotactic activity for various leukocyte classes and subsets [1]. The family of antimicrobial peptides, also called natural antibiotics, constitutes the important immediate effector molecules against invading microorganisms [2, 3]. Accumulating evidence has revealed that the families of chemokines and antimicrobial peptides have substantially overlapping functions. While a number of antimicrobial peptides are chemotactic for selected classes and subsets of leukocyte [4], many chemokines have a substantial microbicidal activity against a broad spectrum of microorganisms [5–7]. Furthermore, CXCL16, a transmembrane-type chemokine [8, 9], was originally identified as a scavenger receptor termed SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein) [10]. Subsequently, a number of chemokines have been shown to display a similar binding activity for typical scavenger receptor ligands including oxidized lipoprotein and bacteria [11]. Thus, the family of chemokines may have substantial functional overlaps with the families of antimicrobial peptides and scavenger receptors. The overlapping functions of these distinct molecular families may have an evolutionary basis stemming from an ancient mode of recognition of pathogens and may represent a certain aspect of the pattern recognition of innate immunity.

The world of antimicrobial peptides

Antimicrobial peptides, now known by >700 in number, are the diverse family of small, mostly cationic polypeptides that have a direct killing activity against bacteria, fungi, parasite, and even some enveloped viruses [2, 3]. Peptides with similar structures and functions are found in virtually all branches of multicellular organisms. Their phylogenic relationships are, however, mostly unclear. This is mostly

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because there has been a strong evolutionary pressure for their gene multiplication and amino acid sequence diversification in order to cope with a wide variety of microorganisms [12–15]. The fundamental structural principal common to most antimicrobial peptides is the topological (rather than linear) amphipathic design, where clusters of hydrophobic and cationic amino acids are organized in discrete surface areas (Fig. 1). It is considered that the amphipathic and highly cationic nature of these peptides allows their selective binding and subsequent disruption of bacterial plasma membrane, which is much more negatively charged than that of host cells [2, 3]. Because of such an electrostatic and physicochemical mode of action, most antimicrobial peptides are only effective at relatively high (micomolar) concentrations and at low salt conditions [2, 3]. In mammals, therefore, the antimicrobial peptides are primarily involved in the barrier protection of various epithelial surfaces that are covered with a low salt body fluid. Some peptides are also involved in the non-oxidative bactericidal activity of leukocytes [2, 3].

For example, Paneth cells, which are present at the bottom of crypts in the small intestine, contain numerous large secretory granules that are discharged into the lumen upon various stimulations. Many components of these granules have potent antimicrobial properties and are likely to protect small intestine from microbial infection and colonization [16]. Paneth cells in humans express only two α-defensins, while mouse Paneth cells express not only more than 20 different α-defensins (also called as cryptdins) but also as many as 7 cryptdin-related sequence (CRS) peptides [13, 17]. CRS peptides represent a family of covalently linked homo- and hetero-dimeric antimicrobial molecules, a feature that may further contribute to their diversity for efficient protection of the gastrointestinal mucosa against enteropathogenic microorganisms [13]. Likewise, the non-oxidative mechanisms of human neutrophils are mediated by antimicrobial peptides and proteins stored within its various cytoplasmic granules [18, 19]. Cathepsin G, azurocidin (also called CAP37), BPI (also called CAP57), and α-defensins are restricted to the primary (asurophil) granules, which also contain myeloperoxidase, elastase, and proteinase 3 [18, 19]. Lactoferrin and hCAP-18 (the precursor of LL-37) are restricted to the neutrophil's secondary (specific) granules [18, 19]. Lysozyme, another antimicrobial molecule, occurs in both primary and secondary granules [18, 19]. Whereas azurophil granule contents are delivered preferentially to intracellular phagolysosomes, the specific granule contents are largely secreted extracellularly [18, 19]. Antimicrobial activity is also detected in natural killer cells and T cells, but the effector molecules that mediate the activity have not been systematically characterized. However, one effector molecule is granulysin, which has been shown to kill Gram-negative bacteria, Gram-positive bacteria, fungi and intracellular *Mycobacterium tuberculosis* [20]. Human cathelicidin LL-37 and α-defensins HNP 1–3 can be additional effector molecules for microbicidal activity of lymphocytes [21].

There is now substantial evidence that supports the vital role of the antimicrobial peptides in the host defense against bacterial infection (Tab. 1). For example,

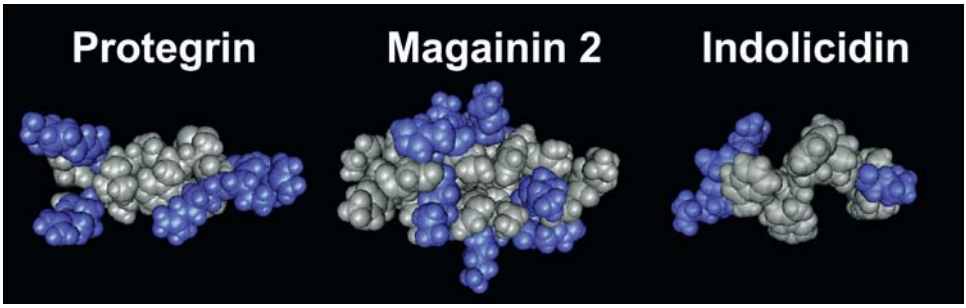

Figure 1
Topological clustering of catinonic and hydrophobic amino acids in antimicrobial peptides. Blue, basic (positively charged) amino acids; gray, hydrophobic amino acids.

the recurrent bacterial infection of lung in patients with cystic fibrosis could be in part due to poor performance of peptide-dependent antibacterial activity in the high-salt bronchotracheal fluid of these patients [22]. The abnormal expression of α-defensins and LL-37 correlates with the occurrence of severe periodontal infectious disease in patients with morbus Kostmann [23]. Mice deficient in the matalloprotease matrilysin, which is necessary to cleave the proforms of epithelial α-defensins in the small intestine, were shown to be more sensitive to orally administered bacteria [24]. Mice with targeted disruption of the cathelicidin gene *Cnlp* displayed a highly elevated susceptibility to Group A *Streptococcus* in a necrotizing cutaneous infection model [25]. Conversely, cathelicidin-resistant mutants of Group A *Streptococcus* demonstrated increased virulence *in vivo*, generating skin lesions of larger size and longer duration in wild-type mice [25]. Importantly, leukocytes derived from cathelicidin-deficient mice were functionally competent in chemotaxis and oxidative burst activity [25]. Thus, the absence of the antimicrobial peptide in the neutrophil granule and epidermal keratinocytes greatly compromises the host innate immunity against Group A *Streptococcus* infection [25]. Collectively, there is now little doubt about the vital role of antimicrobial peptides in innate immunity against invading microorganisms.

Chemotactic activity of antimicrobial peptides

Mammalian defensins and cathelicidins have also been shown to have multiple receptor-mediated effects on immune cells [4]. Most notably, many of them are chemotactic for selective leukocytes and apparently interact with pertussis toxin-sensitive Gαi-coupled seven-transmembrane receptors [4]. In this context, Yang et

Table 1 - In vivo *evidence for the vital role of antimicrobial peptides in host defense against bacterial infection*

Disease or genetic modification	Manifestation	Cause or mechanism	Refs.
cystic fibrosis	recurrent bacterial infection of the lung	high-salt inactivation of peptide-dependent antimicrobial activity	[22]
morbus Kostmann	severe periodontitis	lack of secretion of LL-37 in saliva	[23]
MMP-7 deficient mice	elevated susceptibility to orally administered bacteria	lack of processing of epithelial α-defensins	[24]
cathelicidin-deficient mice	elevated susceptibility to Group A *Streptococcus* skin infection	lack of cathelicidin expression in neutrophils and epithelial cells	[25]
β-defensin 1-deficient mice	poor clearance of *Haemophillus influenzae* in the lung colonization by *Staphylococcus* in the bladder	lack of α-defensin 1 expression in epithelial cells	[51, 52]
human α-defensin-5 transgenic mice	resistance to oral challenge with *Salmonella syphimurium*	transgenic expression of human α-defensin 5 in Paneth cells	[53]

al. have demonstrated that human β-defensins are potent agonists for CCR6 [26], the receptor for a chemokine CCL20/LARC, which is expressed by various epithelial cells and attracts immature dendritic cells and effector lymphocytes [1, 27–30]. In fact, β-defensins appear to have a tertiary structure very similar to that of CCL20 and thus may act as "minichemokines" [31]. Furthermore, LL-37 has been shown to attract neutrophils, monocytes, and mast cells via human formyl peptide receptor-like 1 (FPRL1) [32]. Its angiogenic activity is also mediated by FPRL1 expressed on endothelial cells [26]. While human β-defensins HBD1–3 and mouse β-defensins mBD2 and 3 attract immature dendritic cells via CCR6, HBD3 may also use a receptor other than CCR6 for attraction of monocytes because these cells do not express CCR6 [4]. Human α-defensins HNP1–3 also use an unknown Gαi-protein-coupled receptor(s) because their chemotactic activity can be blocked by pretreatment of target cells with pertussis toxin [4]. Collectively, it is now clear that many antimicrobial peptides can be regarded as endogenous ligands for some Gαi-protein-coupled chemotactic receptors. Thus, besides direct killing of invading microorganisms, antimicrobial peptides may also have an important role in the recruitment of leukocytes in innate and acquired immunity.

Antimicrobial activity of chemokines

Chemokines play pivotal roles in both innate and acquired immunity primarily by inducing directed migration of various leukocyte classes and subsets via interactions with a group of Gαi-protein-coupled seven transmembrane receptors [1]. Furthermore, recent studies have revealed that many chemokines have a direct microbicidal activity (Tab. 2). Krijgsveld et al. determined the amino acid sequences of the purified antibacterial molecules termed thrombocidins that were stored in the α-granules of human platelets [33]. The molecules turned out to be two related chemokine variants processed from a common precursor platelet basic protein (PBP) and truncated by two amino acids in the C terminus, namely, NAP-2/CXCL7(59–126) and CTAP-III/CXCL7(44–126) [33]. The full-length NAP-2/CXCL7(59–128) and CTAP-III/CXCL7(44–128) were not microbicidal in their hands [33]. Tang et al. also characterized antimicrobial molecules released by human platelets upon thrombin stimulation [34]. They demonstrated that several platelet chemokines including CXCL4/PF-4, CCL5/RANTES, the full-length CTAP-III/CXCL7(44–128) and the CTAP-III precursor PBP/CXCL7(35–128) had potent antimicrobial activity against Gram-negative *Escherichia coli*, Gram-positive *Staphylococcus aureus*, *Cryptococcus neoformans*, and, with the exception of CTAP-III and PBP, *Candida albicans* [34]. In their hands, thus, the full-length CTAP-III was also active. Furthermore, Cole et al. examined a panel of 11 chemokines representing all four chemokine subfamilies for antimicrobial activity and demonstrated that the three IFN-inducible non-ELR-motif CXC chemokines, MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, were microbicidal against *Escherichia coli* and Gram-positive *Listeria monocytogenes* [5]. We also reported a broad-spectrum antimicrobial activity of CCL28/MEC (see below) [6], a chemokine selectively expressed by various mucosal tissues [35, 36]. Yang et al., who have originally reported that human β-defensins are functional ligands for CCR6 [26], also tested whether CCL20/LARC was in converse microbicidal [7]. They found that, similar to β-defensins, CCL20 was microbicidal against *Escherichia coli*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Candida albicans* [7]. Furthermore, they demonstrated that many other chemokines also displayed similar antimicrobial activities [7]. These included CXCL1/Gro-α, CXCL2/Gro-β, CXCL3/Gro-γ, CXCL12/SDF-1, CXCL13/BLC, CXCL14/BRAK, CCL1/I-309, CCL8//MCP-2, CCL11/Eotaxin, CCL13/MCP-4, CCL17/TARC, CCL18/PARC, CCL19/ELC, CCL21/SLC, CCL22/MDC, CCL25/ TECK, and XCL1/Lymphotactin [7]. Thus, about two-thirds of the chemokines that were investigated in their study showed the capacity to kill microorganisms *in vitro*. Most bactericidal chemokines, in particular CXCL1, CXCL2, CXCL3, CXCL12, CXCL13, CCL1, CCL13, CCL19, CCL20, and XCL1, were more potent against Gram-negative *E. coli* than against Gram-positive *S. aureus*. A striking difference was observed between the antimicrobial activity of closely related CCL19 and CCL21 [1]. CCL19

Table 2 - Evidence for antimicrobial activity of chemokines

Authors	Source	Target microorganisms	Active chemokines	Inactive chemokines	Refs.
Kriejgsved et al.	Human platelets	*B. subsitis*, *E. coli*, *S. aureus*, *L. lactis*, *C. neoformans*	NAP-2/CXCL7(59-126) CTAP-III/CXCL7(44-126)	Full-length NAP-2(59-128) Full-length CTAP-III(44-128)	[33]
Tang et al.	Human platelets	*E. coli*, *S. aureus*, *C. albicans*, *C. neoformans*	PF-4/CXCL4, RANTES/CCL5, CTAP-III/CXCL7(44-128) PBP/CXCL7(35-128)		[34]
Cole et al.	Recombinant proteins	*E. coli, L. mono-cytogenes*	MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11	IL-8/CXCL8, ENA-78/CXCL5, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, FTN/CX3CL1, LTN/XCL1	[5]
Hoover et al.	Synthetic proteins	*E. coli*, *S. aureus*, *C. albicans*	LARC/CCL20	MCP-1/CCL2	[54]
Hieshima et al.	Recombinant proteins	*C. albicans*, *P. aeruginosa*, *K. pneumoniae*, *S. mutans*, *S. pyogenes*, *S. aureus*	MEC/CCL28	CTACK/CCL27	[6]
	Recombinant proteins	*E. coli*, *P. aeruginosa*, *M. catarrhalis*, *S. pyogenes*, *E. faecium*, *S. aureus*, *C. albicans*	Groα/CXCL1, Groβ/CXCL2, Groγ/CXCL3, SDF-1/CXCL12, BLC/CXCL13, BRAK/CXCL14, I-309/CCL1, MCP-2/CCL8, eotaxin/CCL11, MCP-4/CCL13, TARC/CCL17, PARC/CCL18, ELC/CCL19, LARC/CCL20, SLC/CCL21, MDC/CCL22, TECK/CCL25, LTN/XCL1	GCP-2/CXCL6, IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7, LEC/CCL16, CTACK/CCL27, FTN/CX3CL1	[7]

was active against *E. coli* with little detectable activity against *S. aureus*. On the other hand, CCL21 demonstrated a potent activity against *S. aureus*, while being less potent against *E. coli* than CCL19 [7]. Even though there are some discrepancies concerning antimicrobial activity of some chemokines (Tab. 2), these studies have clearly demonstrated that many chemokines have an intrinsic microbicidal activity when tested in low salt assay conditions *in vitro*.

In particular, CCL28/MEC is expressed at high levels in the mucosal tissues such as salivary glands, trachea, colon, and mammary glands [35, 36]. CCL28 is most homologous with CCL27/CTACK, which is selectively expressed in the skin [37, 38]. These two chemokines commonly act on CCR10 [35, 36, 39, 40]. We observed that CCL28 was not only strongly expressed in the salivary glands but also secreted into the saliva and milk at relatively high concentrations [6]. Furthermore, we noticed that the extended C-terminal regions of CCL28 is highly enriched with histidine residues and shows a significant sequence similarity with histatin-5, a histidine-rich candidacidal peptide secreted in human saliva [6, 41]. These observations led us to examine potential microbicidal activity of CCL28 and its C-terminal peptide. As summarized in Table 3, we found that CCL28 indeed exerts a potent antimicrobial activity against not only *Candida albicans*, but also against Gram-negative bacteria and Gram-positive bacteria [6]. Like histatin-5, the synthetic peptide corresponding to the 28-amino acid C-terminal segment of CCL28 (CCL28-C) also showed a selective antimicrobial activity against *C. albicans* [6]. On the other hand, CCL27, which is most closely related to CCL28 [37, 38], hardly showed such antimicrobial activity [6]. CCL28 rapidly generated pores in the membrane of target microbes [6]. Like many other antimicrobial chemokines and peptides, the microbicidal activity of CCL28 is salt-sensitive [6]. In this context, it should be noted that the mucosal fluids such as saliva, milk, and tracheal and colonic secretions are commonly low in salt concentrations. Thus, CCL28, which is secreted into low-salt body fluids at high concentrations, may have a potential as a direct microbicidal factor. It is also noteworthy that the chemokines with potent antimicrobial activities such as CXCL9, CXCL10, CXCL11, and CCL20 are all expressed and secreted at relatively high concentrations by various epithelial cells [27–30, 42]. Collectively, some chemokines may have a substantial role in host defense against microorganisms as direct microbicidal agents.

Common structural features of chemokines with antimicrobial activity

Like many other antimicrobial peptides, the chemokines with antimicrobial activity tend to have a higher pI than those without such activity, indicating that cationicity is an important feature for antimicrobial chemokines [7]. However, cationicity alone is not sufficient to distinguish chemokines with and without antimicrobial activity. Furthermore, the potency of antimicrobial chemokines does not directly

Table 3 - Summary of antimicrobial activity of CCL28

Microbe	IC_{50} (μM)				
	CCL28	mCCL28	CCL27	CCL28-C	Histatin-5
P. aeruginosa	0.4 ± 0.1	1.7 ± 0.1	>10	>10	>10
K. pneumoniae	0.3 ± 0.1	1.6 ± 0.1	>10	>10	3.0 ± 0.7
S. mutans	1.7 ± 0.4	1.5 ± 0.3	>10	>10	>10
S. pyogenes	3.0 ± 0.2	4.5 ± 0.4	>10	>10	>10
S. aureus	0.9 ± 0.1	0.9 ± 0.1	>10	7.0 ± 1.2	>10
C. albicans	0.7 ± 0.2	1.3 ± 1.0	5.0 ± 1.9	1.6 ± 0.4	3.5 ± 1.6

IC_{50}, 50% inhibitory concentration; mCCL28, mouse CCL28; CCL28-C, the C-terminal 28 amino acid peptide of CCL28

correlate with their cationicity. Therefore, in addition to cationicity, other structural features are necessary for a given chemokine to have an antimicrobial activity [7]. As shown in Figure 2, comparison of the structures between chemokines with and without antimicrobial activities suggests that the topological formation of a large, positively charged electrostatic patch on the surface of the molecule is likely to be a common feature of antimicrobial chemokines. The rest of the molecule is mostly hydrophobic with spotted negative electrostatic charges.

Scavenger receptor activity of chemokines

Scavenger receptors are a highly heterogeneous group of cell surface molecules that commonly bind and internalize oxidized low density lipoprotein (OxLDL) and polyanionic molecules [43]. Scavenger receptors are expressed by myeloid cells (macrophages and dendritic cells) and some endothelial cells, and play an important role in uptake and clearance of modified host molecules, apoptotic cells, microorganisms, and their products [44]. CXCL16, a transmembrane-type chemokine [8, 9], was originally identified as a scavenger receptor for oxidized lipoprotein [10]. CXCL16 is expressed by cells such as macrophages and dendritic cells, and has been shown to bind and internalize various scavenger receptor ligands such as oxidized lipoprotein, bacteria, and sulfated polyanions [10, 45]. Shimaoka et al. have shown that not only CXCL16, but also 12 out of 15 chemokines examined are capable of binding typical scavenger receptor ligands such as OxLDL, Gram-positive bacteria, and Gram-negative bacteria [11]. Furthermore, OxLDL effectively blocks the binding of such chemokines to their respective receptors, suggesting that the receptor binding site of these chemokines mostly overlaps with their potential binding site for

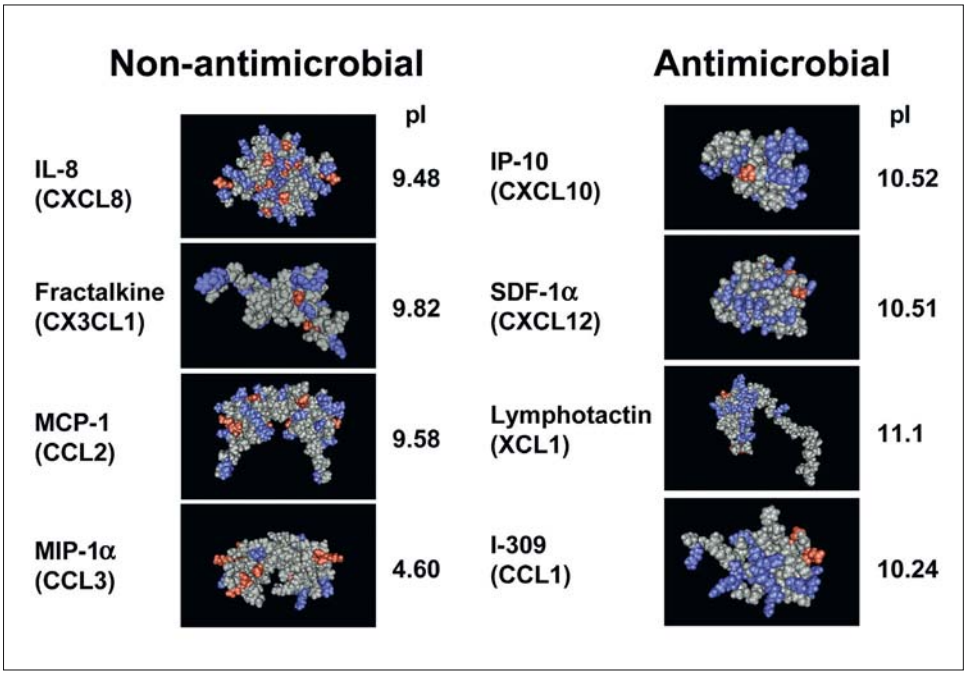

Figure 2
Topological distribution of charged amino acids in non-antimicrobial and antimicrobial chemokines
The pI value of each chemokine is indicated on the right. Red, acidic (negatively charged); blue, basic (positively charged); grey, hydrophobic/neutral.

OxLDL [11]. Indeed, both the chemotactic and scavenger receptor activities of CXCL16 were similarly impaired by a series of mutations in the chemokine domain [11]. As expected, the chemokines with antimicrobial activity consistently bound more avidly with OxLDL and bacteria than those without antimicrobial activity [11].

Concluding remarks

It is now apparent that many chemokines have a potential antimicrobial activity and can also avidly bind OxLDL and other scavenger receptor ligands including bacteria. Thus, chemokines, antimicrobial peptides, and scavenger receptors have some molecular properties in common. The evolutionary origin of such shared properties is not clear but could be related to an ancient pattern recognition of microbial

pathogens by the host [46]. Alternatively, such properties might have been acquired through evolutionary conversion. At any rate, there could have been a strong selective pressure toward retaining and/or acquiring some common molecular features.

The obvious common property of chemokines with antimicrobial peptides and scavenger receptors is cationicity. This could be essential for the antimicrobial peptides and scavenger receptors to recognize bacterial cells that have much higher negative charges than host cells [2, 3]. On the other hand, there may not be such intrinsic functional necessity for chemokine *per se* to be cationic. However, one important reason for most chemokines to be cationic is that the N-terminal regions of the chemokine receptors are highly rich in acidic residues and even sulfated at some tyrosine residues [47, 48]. In fact, many chemoattractant receptors are commonly negatively charged at their N-terminal extracellular domains [48]. Currently, most chemokines are considered to interact with their receptors in a two-step process [49]. The first high-affinity interaction mainly involves the N-terminal region of the receptor and is mostly mediated by strong electrostatic force. The subsequent lower affinity interaction involves other extracellular loops of receptors, while the N-terminal region of chemokines plays a critical role in signaling. Chemokines also interact with negatively charged glycosaminoglycans such as heparin and heparan sulfate, and this property is necessary for their *in vivo* activity [50]. These biological requirements may in part explain the common cationic property of most chemokines. Thus, their possession of antimicrobial and scavenger receptor-like activities may be mostly fortuitous (a matter of *in vitro* assays) but may still have some physiologic implications for some chemokines.

At present, the antimicrobial activity of chemokines has been shown only by *in vitro* assays. Thus, studies using knockout mice or transgenic mice would be necessary to prove any physiologic role of chemokines in direct microbial killing *in vivo*. Given the micromolar concentrations required for effective microbicidal activity, however, it is unlikely that direct killing of microorganisms is a major function of any chemokines. However, still some chemokines may play a significant role in direct killing of microorganisms through cooperation with other chemokines and other antimicrobial peptides. In contrast, the chemotactic activity of antimicrobial peptides are more physiologically attainable, requiring only nanomolar concentrations [4]. Furthermore, there could still be a large number of new antimicrobial peptides that remain to be characterized. For example, an improved genome-wide search has recently identified a total of 28 new human and 43 new mouse β-defensin genes that are clustered in five syntenic chromosomal regions [15]. Thus, it is quite a challenge to characterize such new peptides for their antimicrobial spectrum and chemotactic activity, and to identify their chemotactic receptors.

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