

# THE USE OF ANTIMICROBIAL PEPTIDES IN OPHTHALMOLOGY: AN EXPERIMENTAL STUDY IN CORNEAL PRESERVATION AND THE MANAGEMENT OF BACTERIAL KERATITIS

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## ABSTRACT

**Purpose:** Bacterial keratitis is an ocular infection with the potential to cause significant visual impairment. Increasing patterns of antibiotic resistance have necessitated the development of new antimicrobial agents for use in bacterial keratitis and other serious ocular infections. With a view to exploring the use of novel antimicrobial peptides in the management of ocular infection, we performed a series of experiments using synthetic antimicrobial peptides designed for the eradication of common and serious ophthalmic pathogens.

**Methods:** Experiments were performed with three clinical ocular isolates—*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*—in three experimental settings: (1) in vitro in a controlled system of 10 mM sodium phosphate buffer, (2) in vitro in modified chondroitin sulfate-based corneal preservation media (Optisol), and (3) in an in vivo animal model (rabbit) simulating bacterial keratitis. In all cases, outcomes were measured by quantitative microbiological techniques.

**Results:** The candidate peptides (CCI A, B, and C and COL-1) produced a total reduction of the test pathogens in phosphate buffered saline. In modified Optisol, the peptides were effective against *S epidermidis* at all temperatures, demonstrated augmented activity at 23°C against the gram-positive organisms, but were ineffective against *P aeruginosa*. The addition of EDTA to the medium augmented the killing of *P aeruginosa* but made no difference in the reduction of gram-positive organisms. In an in vivo rabbit model of *Pseudomonas keratitis*, COL-1 demonstrated neither clinical nor microbicidal efficacy and appeared to have a very narrow dosage range, outside of which it appeared to be toxic to the ocular surface.

**Conclusions:** Our data indicate that the antimicrobial peptides we tested were effective in vitro but not in vivo. In an age of increasing antibiotic resistance, antimicrobial peptides, developed over millions of years as innate defense mechanisms by plants and animals, may have significant potential for development as topical agents for the management of severe bacterial keratitis. However, modifications of the peptides, the drug delivery systems, or both, will be necessary for effective clinical application.

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The emergence of multiply drug-resistant bacteria . . . would represent the most important issue in antibiotic resistance since the dawn of the antibiotic era. A common virulent and transmissible bacterial agent with no known effective therapy would set infectious diseases back 60 years.

*Annals of Internal Medicine*, 1996<sup>1</sup>

Cationic peptides have been found in all forms of life from bacteria to man and are probably the most conserved theme in nature's struggle to control aggressive microorganisms.

*Drugs*, 1997<sup>2</sup>

Most species throughout the evolutionary scale use peptides as antimicrobial agents. It is likely that resistance to peptide antibacterial agents may not develop easily. Since the problem of antibiotic resistance is presently a particularly severe one, peptide antibiotics may be the drugs of choice in the future.

*Biochimica et Biophysica Acta*, 1994<sup>3</sup>

[Peptide antibiotics] might be ideal therapeutic agents, avoiding the problem of acquired resistance.

*Nature*, 1997<sup>4</sup>

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## INTRODUCTION

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Ocular infections involving the optical media of the eye or the neurosensory retina may have profound and devastating impact on visual function. Pathogenic invasion of the cornea or the internal eye always carries the risk of significant functional visual damage because of (1) the small space in which the infection occurs, (2) structural disruption of the optics of the cornea in the case of keratitis, or (3) the rapid and irreversible destruction of neuroretinal tissue in the case of endophthalmitis. While the treatment of corneal ulcers with topical antimicrobial agents has been notably successful with an expanding array of both focused and broad-spectrum antibiotics, there has, in general, been an alarming emergence of patterns of increasing resistance to commonly used antimicrobial agents.<sup>5-9</sup> Microbes cleverly develop resistance to antibiotics as a result of chromosomal mutation, inductive expression of a latent chromosomal gene, or exchange of genetic material via transformation, bacteriophage transduction, or plasmid conjugation.<sup>5,10</sup>

Use of the fluoroquinolones in the management of external infections is the most recent example of how a new class of antibiotics has been instrumental in changing management strategies for the treatment of corneal infections. Nonetheless, emerging patterns of resistance even to these new classes of antimicrobial agents<sup>11-25</sup> have stimulated the continuing quest for an agent that provides rapid and complete microbicidal activity with minimal toxic effects and susceptibility to mechanisms of microbial resistance.

The problem of emerging antimicrobial resistance and the need to find more effective antimicrobial agents stimulated us to initiate investigation of antimicrobial peptides as a tool for the management of ocular infection. Indeed, the innate gene-encoded antimicrobial peptides are increasingly being recognized as host defense effector molecules in plants and animals,<sup>26</sup> and since they differ structurally from conventional antibiotics produced by bacteria and fungi, they may offer novel templates for pharmaceutical compounds that could be used against increasingly resistant microbes.<sup>27</sup> This thesis presents the results of a series of *in vitro* and *in vivo* experiments performed in our laboratory in an effort ultimately to expand the armamentarium of effective antimicrobial agents for the management of severe microbial keratitis.

## BACKGROUND: THE ANTIMICROBIAL PEPTIDES

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The defense system of the eye consists of both general anatomical and specific immune responses to microbial invasion. The lids and cilia represent the first protective mechanism against pathogenic invasion. The tear film is,

likewise, an important defense against microbial invasion, both for its flushing function and its composition, which includes immunoglobulins, lysozyme, lactoferrin,  $\beta$ -lysin, and other proteins with antimicrobial capabilities.<sup>28-31</sup> These defenses notwithstanding, a breach of the corneal epithelial barrier by a pathogenic organism can render the cornea defenseless against the destructive mechanisms of a virulent pathogen. In such cases, infection management requires the application of an antimicrobial agent.

Ophthalmic researchers have paid relatively little attention to the emerging field of peptide chemistry as a tool to augment the anatomical and specific immune responses of the eye to pathogenic invasion. Yet, for the past two decades, workers have been fascinated with the cellular immune defense mechanisms elaborated by organisms in response to pathogenic infection, and for at least three decades, interest in endogenous peptides with antimicrobial properties has increased.<sup>26,32,33</sup> These peptides are part of the innate immune response to pathogenic infection that has developed throughout nature. The range of antimicrobial peptide research encompasses subject matter far too broad for the scope of this thesis. However, definition and categorization of the peptides with antimicrobial activity are necessary for consideration of the current experimentation.

## TERMINOLOGY, STRUCTURE, AND CLASSIFICATION

The terminology applied to these antimicrobial substances varies in the scientific literature. Descriptive terms that have been used include "defense peptides," reflecting their teleological or functional role in defense against microbial invasion; "lytic peptides" or "pore-forming proteins," reflecting their probable action as membrane-permeabilizing agents; "cationic peptides," reflecting their electrochemical structure; and "antimicrobial peptides," a more generic term describing their functional capabilities. For the purposes of this presentation, we will use the more generic term—antimicrobial peptides.

Antimicrobial peptides are small, basic, single gene-encoded peptides that are generally synthesized as preproteins and are activated as part of the host defense systems in plants,<sup>34,35</sup> insects,<sup>36-41</sup> fish, amphibia,<sup>42-44</sup> birds, and mammals.<sup>45-47</sup> These small proteins are an evolutionarily ancient system of immune protection that are expressed during infection, inflammatory events, and even wound repair, and their presence constitutes a key innate host defense against microbial pathogenesis.<sup>48-51</sup> Their *de novo* synthesis or release from storage sites can be induced extremely rapidly, making them particularly important in the initial phases of resistance to microbial invasion, and current scientific evidence demonstrates that they function as membrane permeabilizing agents.<sup>47</sup>

Antimicrobial peptides are produced ubiquitously throughout nature. Many of these relatively short peptides (12 to 50 residues) are lethal to bacteria, fungi, and parasites<sup>52</sup> but display minimal toxic effects on mammalian cells. Although impressively diverse in structure, most antimicrobial peptides are highly cationic (positively charged) and amphipathic. This electrochemical structure facilitates their binding to negatively charged biological membranes on which they aggregate and act as lytic pore formers.<sup>45,47,53</sup> The lytic peptides, both those with  $\alpha$ -helical and those with  $\beta$ -pleated sheet structures, are amphipathic; that is, one side of the molecule is hydrophilic and one side is hydrophobic, a design that is consistent with membrane-specific interaction.<sup>54</sup>

The origin of synthesis of the antimicrobial peptides varies according to the host species. In insects, the fat body is the primary location of peptide synthesis. In the frog, the skin is the location of synthesis. Human and other mammalian defensins are expressed in granulocytes and are formed during early hematopoiesis; they may also be found in tracheal and lung macrophages and in the Paneth cells of the small intestine.

On a chemical and biochemical basis, the antimicrobial peptides can be divided into four families:

- Cysteine-rich peptides that form amphiphilic  $\beta$ -pleated structures with two or more disulfide bonds (eg, defensins, tachyplesins, protegrins (Table IA))
- Linear molecules without cysteine (Cys) in the form of  $\alpha$ -helical peptides (eg, cecropins and magainins [both amphipathic helices], bombinins) (Table IB)
- Molecules with one disulfide bond or cysteine-disulfide ring peptides (eg, bactenecins [bovine cyclic dodecapeptides], brevinins, ranalexin) (Table IC)
- Peptides with an overrepresentation of one or two amino acids (eg, Pro, Arg, Trp, Gly): apidaecins, indolicidin, drosocin, PR-39 (Table ID)<sup>45,46</sup>

A notable characteristic of all the antimicrobial peptides is that they have well-defined tridimensional structures (secondary structure). The function of each peptide is dependent to a great extent on this conformational structure, which is specified by the amino acid sequence (primary structure), the presence or absence of disulfide bonds, and the variable terminal portions of the molecules.<sup>55,56</sup>

One can also classify the pore-forming defense peptides by their species of origin (Table II).

#### MECHANISM OF ACTION

The antimicrobial peptides, produced ubiquitously throughout nature, function as “natural” antibiotics through the mechanism of pore formation—permeabilizing and disrupting the biological membranes of target cells. These peptides, often in aggregate form, insert into cell membranes, making the target cells leaky and

ultimately killing them<sup>47,57-59</sup> (Figure 1).

The clonally based immune system alone would not be sufficient to stave off bacterial infection. It is important to recall that bacteria can double in 20 minutes, while responsive lymphocyte induction may take many hours. Therefore, throughout the evolutionary scale, multiple species from insects to mammals have developed a “rapid response” system consisting of lytic peptides that can be synthesized and excreted and that act directly and rapidly on microbial pathogens.<sup>54</sup>

The mechanism of pore formation as a strategic solution has evolved over millions of years, beginning with primitive organisms and evolving through the higher vertebrates. Primitive eukaryotes, such as *Entamoeba histolytica*, are known to elaborate pore-forming agents that allow them to kill on contact, as do simple prokaryotes. Bacteria can produce pore-forming peptides as well, an example of which are the “hemolysins,” so designated because of their ability to lyse erythrocytes. These pore-forming agents may be required for the pathogenesis of organisms, and it is through the activity of the pore-forming substances that these organisms produce clinical disease. Table III includes some examples of pore-forming agents produced by bacteria that cause disease in humans.

The mechanism of pore formation differs among various peptides. Defensins, for example, are cationic proteins that form a triple-stranded,  $\beta$ -pleated sheet at one end and a hydrophobic finger at the other. The initial contact between the target lipid cell bilayer is thought to be between the cationic arginine groups on the defensin molecule and the negatively charged target membrane. This is followed by the formation of defensin multimers, creating a channel that spans the membrane, leading to membrane permeabilization and disruption.<sup>45,60,61</sup> Similarly, the  $\alpha$ -helical cecropins bind to the target membrane electrostatically, undergo a process of multimerization, and then form membrane-spanning pores permeabilizing the outer and inner membranes of target bacteria<sup>45,62,63</sup> (see Figure 1).

A good example of the way in which peptide-induced pore formation occurs is the interaction with gram-negative bacteria.<sup>64</sup> The cell envelope of a gram-negative bacterium is composed of two membrane systems, the outer of which contains negatively charged lipopolysaccharide molecules.<sup>3</sup> Cationic antimicrobials bind to this outer lipopolysaccharide membrane and disrupt its structure. When the inner membrane is encountered, the cationic peptides form channels, altering membrane permeability. This interaction with the outer membrane of gram-negative bacteria has been confirmed for magainins, defensins, cecropins, bactenecins, and tachyplesins, among others.<sup>3</sup>

Of the substances included in the previous discussion, we will focus on a select few that have been thoroughly

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**TABLE IA: CYSTEINE-RICH AMPHIPHILIC  $\beta$ -PLEATED PEPTIDES**

PEPTIDE	STRUCTURE	SIZE	SPECIES	TISSUE SOURCE	SPECTRUM
Defensins	6 cysteines 3 C-C bridges arginine-rich	29-45 amino acids	Mammals, insects, birds, plants	Leukocyte granules, Paneth cell granules, fat bodies (insects), plant seeds	Gram+/- bacteria, fungi, enveloped viruses
Protegrins	COOH-terminal amide	16-18 amino acids	Pig	Leukocytes	Gram+/- bacteria, fungi, enveloped viruses
Tachyplesins	4 cysteines 2 C-C bridges	16-18 amino acids	Horseshoe crab	Hemocyte granules	Gram+/- bacteria, fungi, enveloped viruses

**TABLE IB: AMPHIPHILIC  $\alpha$ -HELICAL PEPTIDES**

PEPTIDE	STRUCTURE	SIZE	SPECIES	TISSUE SOURCE	SPECTRUM
Magainins	Lysine-rich	20-27 amino acids	Frog	Skin (granular gland and intestinal tract)	Gram+/- bacteria, fungi, parasites
Cecropins	Lysine-rich terminal amide	34-45 amino acids	Insect	Hemolymph, hemocytes, fat body	Gram+/- bacteria, fungi, parasites

**TABLE IC: CYSTEINE-DISULFIDE RING PEPTIDES**

PEPTIDE	STRUCTURE	SIZE	SPECIES	TISSUE SOURCE	SPECTRUM
Cyclic dodecapeptide	Arginine-rich No amphiphilic tail	12 amino acids	Bovine	Granulocytes	Gram+/- bacteria
Ranalexin	C-C bridged ring COOH-terminal	20 amino acids	Frog	Skin	Gram+/- bacteria
Brevinins	C-C bridged ring COOH-terminal	24-30 amino acids	Frog	Skin	Gram+/- bacteria

**TABLE ID: LINEAR PEPTIDES WITH A PREDOMINANCE OF AMINO ACID(S)**

PEPTIDE	STRUCTURE	SIZE	SPECIES	TISSUE SOURCE	SPECTRUM
Indolicidin	Tryptophan-rich	13 amino acids	Bovine	Granulocytes	Gram+/- bacteria
PR-39	Proline- and arginine-rich	39 amino acids	Pig	Small intestine leukocytes	Gram+/- bacteria

characterized and have been of some direct relevance to ophthalmic applications. These include the magainins, the defensins, and the cecropins.

### MAGAININS

The magainins were first reported in 1987 by Zasloff, who was attempting to find the agent to explain the curious lack of infection in the healing surgical wounds in the frog *Xenopus laevis*.<sup>42,65-67</sup> These frogs developed infection very rarely, even when they had open, healing wounds and

were kept in contaminated containers. Zasloff isolated and characterized the first of these peptides located in the skin of the frog. He called them magainins 1 and 2, after the Hebrew word *magain* (shield), since they appeared to shield the frogs from infection. Since that time, the magainins have been characterized as a family of at least a dozen ionophoric, linear, cationic amphipathic peptides, 21 to 27 amino acids in length and generally lacking cysteine.<sup>68,69</sup> The magainins are produced in the granular glands and stored in secretory vesicles, and they have a

TABLE II: SELECTED ANTIMICROBIAL PEPTIDES CLASSIFIED BY SPECIES ORIGIN

SPECIES	PEPTIDE
Amphibian ( <i>Xenopus laevis</i> )	Magainin Brevinin
Other amphibians	Dermaseptin Bimbinin
Insect	Cecropin Andropin Sarcotoxin Sapecin Apidaein Abaecin Hymenoptaecin Bee defensin Melittin Attacins
Mammals (rabbit, rat, guinea pig, mouse, human, cow)	Defensin Beta-defensin Indolicidin Bactenecin Azurocidin
Crustaceans (Horseshoe crab)	Tachyplesin

broad spectrum of activity against a range of gram-positive and gram-negative bacteria, fungi, and protozoa. They have also been isolated in the gastric mucosa of the frog.<sup>70</sup> They appear to serve a physiological role in defense against macroscopic predators and in the control of microbial infection following wounding.<sup>26</sup>

The magainins are highly selective, channel-forming, lytic agents that form permeabilizing membrane channels with increasing peptide concentration.<sup>69,71-85</sup> A common

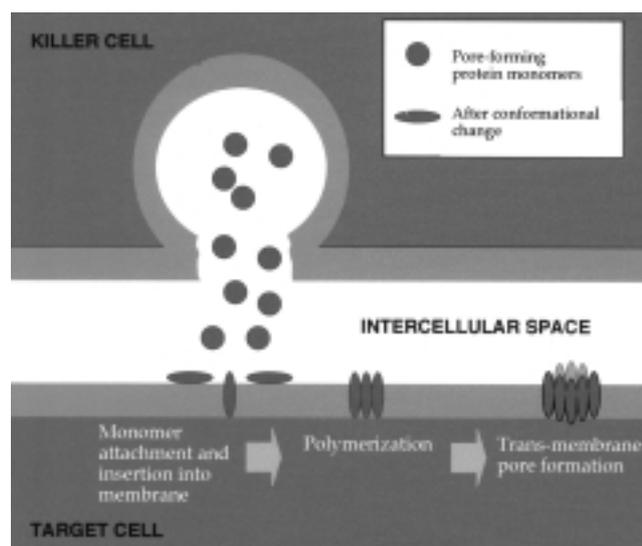


FIGURE 1

Diagrammatic representation of pore formation by antimicrobial peptides in a target cell.

TABLE III: PORE-FORMING AGENTS PRODUCED BY PATHOGENIC BACTERIA

BACTERIA	PORE-FORMING AGENT
<i>Bordetella pertussis</i>	Cytolysin
<i>Clostridium perfringens</i>	Perfringolysin
<i>Escherichia coli</i>	Alpha-hemolysin
<i>Listeria monocytogenes</i>	Listeriolysin O
<i>Pseudomonas aeruginosa</i>	Cytotoxin
<i>Staphylococcus aureus</i>	Alpha-toxin
<i>Streptococcus pneumoniae</i>	Pneumolysin
<i>Streptococcus pyogenes</i>	Streptolysin O
<i>Vibrio cholerae</i>	Hemolysin

structural feature of the magainins and similar peptides is a net positive charge due to the presence of multiple arginine and lysine residues; these amphipathic structures appear to function by binding to anionic phospholipids in the target membranes.<sup>47</sup>

The magainins exhibit a broad spectrum of antimicrobial activity, including activity against gram-positive and gram-negative bacteria, fungi, and protozoa.<sup>42,69,86-89</sup> In addition, they show selective lytic activity against a variety of transformed cells, such as human cancer cells at concentrations tenfold lower than those needed to lyse normal cells.<sup>90</sup> The magainins are the first of the antimicrobial peptides to be harnessed by the pharmaceutical industry for clinical application.

#### DEFENSINS

Stimulated neutrophils have two mechanisms for producing cellular injury. The first depends on the production of reactive oxygen intermediates, such as hydrogen peroxide, that can lyse target cells. The second mechanism is nonoxidative and is mediated by protein cytotoxins that are lodged in the cytoplasmic granules; among these are cathepsins, elastase, and defensins.<sup>91,92</sup> These mammalian defensins are small (3,000 to 4,000 daltons) cysteine- and arginine-rich antimicrobial peptides, approximately 29 to 34 amino acids in length; they contain three disulfide bonds, giving them a complex tertiary folded structure.<sup>93-97</sup> They are isolated from the azurophil granules of mammalian alveolar macrophages and neutrophils, make up about one third to one half of the total protein content of the neutrophil granules in the cells,<sup>98</sup> and constitute the major component of the oxygen-independent antimicrobial pathway of these phagocytic cells.<sup>48,98-102</sup>

Neutrophil defensins, whose structure is genetically determined, are synthesized by myeloid precursor cells in the bone marrow and are stored in the cytoplasmic azurophil granules of the mature cells.<sup>98</sup> Defensins are delivered to microbial targets after phagocytosis of an invading pathogen when the phagosomes and the azurophil granules within the neutrophil fuse.<sup>45</sup>

Originally termed lysosomal cationic peptides in rabbit and guinea pig polymorphonuclear leukocytes, crude defensin extract accounted for most of the antimicrobial activity against group D *Streptococcus*, *Proteus vulgaris*, *S aureus*, *S epidermidis*, *Candida parapsilosis*, and *Cryptococcus neoformans*. Since the original description, six defensin molecules have been isolated and purified from rabbit neutrophils<sup>101,102</sup> and also have been demonstrated in rats,<sup>103,104</sup> guinea pigs,<sup>93</sup> and humans,<sup>48,60,94</sup> where they constitute up to 7% of the total protein content of phagocytic cells (neutrophils and alveolar macrophages). The amino acid sequences of the defensins are highly conserved across species.<sup>53,93,98,105-109</sup> Table IV demonstrates the sequence of the major mammalian defensins and the remarkable homology between the peptides across mammalian species. Most of these effector protein molecules share significant structural and functional similarities, a finding that suggests their antiquity and conservation over millions of years. Defensins are, however, not limited to mammals. They have also been identified in insects.<sup>110</sup> In addition,  $\beta$ -defensins, which were discovered and isolated from bovine neutrophils, have a distinctly different structure but retain antibacterial properties similar to the defensins.<sup>111</sup>

Initially thought to be confined to cells of myeloid lineage, defensins have now been localized to other tissues. Although the largest quantity of defensins are isolated from phagocytic cells, they can also be found in bovine tracheal cells (TAP-tracheal antimicrobial peptide)<sup>112,113</sup> and in mouse intestinal cells (cryptidins).<sup>114-117</sup>

Like the magainins, the defensins appear to lyse target cells by pore formation.<sup>61,118</sup> The arginine residues associate electrostatically with the anionic portions of the target lipid membrane. These proteins then aggregate, insert into the membrane, and form a permeabilizing pore that leads to the death of the target cell.<sup>60,61</sup> The cytolytic

activity of the defensins against bacteria is extremely ion-sensitive, being greater in media of low ionic strength that lack significant concentrations of calcium or magnesium. In addition, defensin activity is dependent on pH and temperature.<sup>98</sup>

Defensins are broad-spectrum microbicides with demonstrated in vitro activity against gram-positive and gram-negative bacteria, mycobacteria, *Treponema pallidum*, and certain fungi<sup>48,119-124</sup> and enveloped viruses (herpes simplex, vesicular stomatitis virus, and influenza virus).<sup>48,102,120,121,125-129</sup>

Aside from their antimicrobial activity, specific defensins appear to have different functions. These functions include cytotoxicity,<sup>91,118,130-132</sup> chemotactic activity for monocyte recruitment,<sup>133,134</sup> inhibition of corticosteroid production,<sup>135,136</sup> release of histamine from mast cells,<sup>109</sup> augmentation of macrophage phagocytic capacity,<sup>137</sup> inhibition of protein kinase,<sup>138</sup> acceleration of wound healing,<sup>139</sup> and mitogenic effects on epithelial cells and fibroblasts.<sup>140</sup>

#### CECROPINS

Cecropins are natural antimicrobial peptides produced in a variety of insects in response to microbial infection.<sup>141</sup> First isolated from the hemolymph of *Hyalophora cecropia*, the giant silk moth, cecropins were identified as the chief component of the moth's humoral defense system against microbial infection by Boman, Merrifield and colleagues.<sup>141-146</sup> Within hours of injury or infection, a biologically active peptide is induced and is found in the insect hemolymph.<sup>147</sup>

Initially, two distinct cecropin molecules were identified (cecropins A and B).<sup>142</sup> Later, an additional five antimicrobial proteins were identified (cecropins C, D, E, F, and factor G).<sup>148</sup> The cecropins are a family of linear cationic peptides that are between 35 and 37 amino acids

TABLE IV: THE MAMMALIAN DEFENSINS

SPECIES	DEFENSIN	SEQUENCE
Human	HNP-1	ACYCRIPACIAGERRYGTCTIYQGRWAFCC
	HNP-2	CYCRIPACIAGERRYGTCTIYQGRWAFCC
	HNP-3	DCYCRIPACIAGERRYGTCTIYQGRWAFCC
Rabbit	NP-1	VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR
	NP-2	VVCACRRALCLPLERRAGFCRIRGRIHPLCCRR
	NP-3a	GICACRRRFPNSERFSGYCRVNGARYVRCCSRR
	NP-3b	GRCVCRKQLLCSYRERRIGDCKIRGVRFPPCCPR
	NP-4	VSCTCRRFSCGFGERASGSCTVNGVRHTLCCRR
	NP-5	VFCTCRGFLCGSGERASGSCTVNGVRHTLCCRR
Guinea pig	GPNP	RRCICTTRTCRFPYRRLGTCIFQNRVYTFCC
Rat	RatNP-1	VTCYCRTRCGFRERLSCACGYRGRYRLCCRR
	RatNP-2	VTCYCRSTRCGFRERLGGACGYRGRYRLCCRR
	RatNP-3	CSCRTSSCRFCERLSGACRLNGRIYRLCC
	RatNP-4	ACYCRIGACVSGERLTGACGLNGRIYRLCCRR

Amino acid key: A=alanine; C=cysteine; D=aspartic acid; E=glutamic acid; F=phenylalanine; G=glycine; H=histidine; I=isoleucine; K=lysine; L=leucine; N=asparagine; P=proline; R=arginine; S=serine; T=threonine; V=valine; W=tryptophan; Y=tyrosine.

in length.<sup>141</sup> They are synthesized as preproteins of approximately 62 to 64 residues; these are then cleaved into the smaller active molecule.<sup>149</sup> The three principal cecropins are highly homologous and are identified as cecropins A, B, and D.<sup>148</sup> Related cecropin analogues have now been identified in a variety of insect species.<sup>32,141</sup>

The cecropins function by disrupting the cell membrane of target cells.<sup>141,150,151</sup> They are organized such that the first 11 amino acids form a highly amphipathic  $\alpha$ -helix with hydrophobic and positively charged surfaces.<sup>53,145</sup> At the N terminal of the  $\alpha$ -helix, the hydrophilic residues are located on one side of the molecule with the hydrophobic residues on the opposite side, creating the amphipathic structure.<sup>152,153</sup> These molecules have been shown to display pore-forming characteristics and have striking selectivity for prokaryotic rather than eukaryotic cells.<sup>154</sup>

Cecropins and cecropin analogues have a broad spectrum of activity, including gram-positive (eg, *Bacillus* species) and gram-negative (eg, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Acinetobacter calcoaceticus*) bacteria as well as fungi and parasites.<sup>52,142,143,155-160</sup> In addition to their microbicidal activity, the cecropins and synthetic analogues demonstrate markedly increased cytolytic activity against transformed cells (eg, tumor cells) as opposed to normal cells.<sup>161,162</sup>

## **ANTIMICROBIAL PEPTIDES AND OPHTHALMOLOGY**

The application of peptide antimicrobial agents in ophthalmology has been limited, although the theoretical promise of these agents in the management of corneal infection is great, given the accessibility of drug to the site of infection, rapid action, zwitterionic character for transport in biphasic corneal tissues, and potential for well-tolerated, high concentrations at the cornea. In addition, theoretically, the presence of active antimicrobial proteins such as lysozyme and lactoferrin on the ocular surface suggests that this surface has a rather low level of proteolytic enzyme activity. Moreover, the corneal epithelium is negatively charged, a circumstance that should enhance the activity of the positively charged peptide molecules. However, the majority of research on the antimicrobial peptides has remained in the sphere of structure and function, with only a relatively limited effort focused on clinical application.

For more than a decade, our laboratory has investigated the effectiveness of a variety of peptides on ocular pathogens as well as their use in the prevention of contamination in ophthalmic systems. In the following paragraphs, we will review the work that has been done with antimicrobial peptides both in our laboratory and in other centers.

### **DEFENSINS**

In 1988, the Cornea Research Laboratory in collaboration

with the Dairy Food and Safety Laboratory at the University of California, Davis, initiated the defense peptide project to explore ophthalmic applications for the defensins. The defensins constitute candidates with great potential as potent, broad-spectrum, natural antimicrobial agents. Their size, structure, and biochemical configuration suggest that they would be prime candidates for synthetic reproduction and use as biocidal agents.

Cullor, Mannis, and colleagues<sup>127,163</sup> demonstrated the effectiveness of two rabbit defensins, NP-1 and NP-5, against isolates from clinical ocular microbial infections in humans and horses. They showed for the first time the effective microbicidal activity of NP-1 (10  $\mu\text{g}/\text{mL}$ ) against all ocular pathogenic isolates tested (*Candida albicans*,  $\alpha$ -hemolytic *Streptococcus*, *Streptococcus pneumoniae*, *P aeruginosa*, and *Morganella morgani*), effecting a 2- to 3-log<sub>10</sub> (99% to 99.9%) reduction within a 60-minute incubation. NP-5, however, differed markedly, having little bactericidal activity but significant bacteriostatic activity against the isolates tested.

Mannis and colleagues<sup>164</sup> investigated the efficacy of NP-1 for antimicrobial activity against *S aureus*, *P aeruginosa*, and *S pneumoniae* in modified corneal storage media (Optisol without antibiotics) at 4°C, 23°C, and 37°C and demonstrated that at 100  $\mu\text{g}/\text{mL}$ , NP-1 successfully reduced *S pneumoniae* and *S aureus* at all temperatures, while a higher level (200  $\mu\text{g}/\text{mL}$ ) was required for killing *P aeruginosa*, suggesting that the defensin might be a potential additive for the prevention of contamination of corneal storage media.

Murphy and colleagues<sup>140,165</sup> demonstrated that the rabbit defensin NP-1 possesses significant growth factor activity in a serum-free in vitro cell culture system utilizing lens and corneal epithelial cells, suggesting that at the same concentrations at which NP-1 exhibits maximal antibacterial effects, it may also promote epithelial cell growth. This finding stimulated interest in the notion that this substance might perform two functions—antimicrobial debridement and the promotion of wound healing.

### **CECROPINS**

The cecropins have been investigated in a variety of contexts and combinations in ophthalmology.

Gunshefski and colleagues<sup>159</sup> first demonstrated the antimicrobial activity of cecropin analogs against isolates from clinical ocular microbial infections in humans. In this in vitro experiment, the investigators demonstrated greater than 3-log reduction of a panel of test pathogens, including *P aeruginosa*, *S aureus*, *S pneumoniae*, and *C albicans*, with exposure of the organisms to Shiva-11, a synthetic cecropin analogue, in the range of 10 to 100  $\mu\text{g}/\text{mL}$ . They demonstrated dose-dependent effectiveness of the

cecropin analogue against the test panel and found that solutions containing divalent cations appeared to diminish antimicrobial activity of the peptide. They theorized that the cation stabilized cell membranes, thereby inhibiting the activity of the peptide.

The same synthetic analogue was studied by Mannis and colleagues<sup>166</sup> as an antibacterial agent in preservative-free timolol and contact lens solutions. The investigators demonstrated that Shiva-11 effected greater than a 2.5-log reduction of test pathogens, including *P aeruginosa*, *S epidermidis*, and *S aureus*, in either buffered saline containing a contact lens or in preservative-free timolol, and they suggested its use as a novel ophthalmic preservative.

De Sousa and colleagues<sup>55,167</sup> evaluated a cecropin analogue (D<sub>5</sub>C) to compare the antimicrobial efficacy of the peptide against *P aeruginosa* with that of commercially available contact lens disinfecting solutions. The investigators inoculated various concentrations of bacteria into the contact disinfecting solutions and into buffered saline as a control. Samples were incubated, and at various time points, aliquots were removed and were plated and subcultured on nutrient agar. At 72 hours, all contact lens solutions tested produced greater than a 2-log reduction of the organism. However, the addition of D<sub>5</sub>C to the contact lens solutions yielded greater than 3 logs killing with a larger inoculum of bacteria and with a contact lens in the solution. The investigators demonstrated that D<sub>5</sub>C effectively augmented antimicrobial activity of the disinfecting solutions.

Schwab and colleagues<sup>168</sup> examined the effectiveness of two peptides (D<sub>5</sub>C and Nisin) against *P aeruginosa*, *S epidermidis*, *S pneumoniae*, and *C albicans* in modified corneal storage media (Optisol without antibiotics). The investigators were unable to demonstrate peptide activity in any of the testing situations at either 4°C or 27°C, although the addition of EDTA augmented killing of *P aeruginosa* in the test system.

In an extensive study of the cecropin analog D<sub>5</sub>C, de Sousa and colleagues<sup>169</sup> evaluated the efficacy of the peptide in both contact lens sterilization and corneal storage media against *P aeruginosa*, *Serratia marcescens*, *S aureus*, *S epidermidis*, *S pneumoniae*, and *C albicans*. She concluded the following:

- In concentrations greater than 5 µg/mL, the peptide demonstrated antimicrobial activity against all the pathogenic species tested, with greater than a 3-log reduction after 30 minutes of exposure to the peptide in vitro in phosphate buffered saline.
- Antimicrobial activity was dose- and exposure-dependent in phosphate buffered saline.
- At the concentration of 100 µg/mL, D<sub>5</sub>C demonstrated significant antimicrobial activity against the panel 24 hours after exposure and augmented the activity of commercial solutions.
- The peptide did not demonstrate antimicrobial activity

against the test panel in corneal preservation media (Optisol) independent of the temperature.<sup>55</sup>

Gunsheski and colleagues<sup>169</sup> demonstrated that the cecropin analog Shiva-11 (100 µg/mL) was effective against highly gentamicin-resistant organisms in vitro. Gruzensky and colleagues<sup>170</sup> evaluated the effectiveness of a synthetic cecropin analog (Hecate) in an in vitro culture of *Acanthamoeba* species (*A castellani* and *A polyphaga*). This study compared the cecropin analogue with the anti-amoebic activity of bovine neutrophil peptide (BNP-1), propamidine isethionate (Brolene), and neomycin, and demonstrated the cysticidal effect of the cecropin analogue between 250 and 500 µg/mL, with partial inhibition of organisms down to 50 µg/mL.

Murphy and colleagues<sup>171</sup> demonstrated that the cecropin analogue (Shiva-11) was mitogenic for corneal epithelial cells and fibroblasts in culture. This finding that cecropin, in specific dose ranges, possesses growth factor activity in a serum-free in vitro cell culture system suggested that it might be useful as both an antimicrobial and a wound-healing agent.

Nos-Barbera and colleagues<sup>186</sup> used an experimental rabbit model of *Pseudomonas keratitis* to investigate the antimicrobial characteristics of a hybrid peptide consisting of cecropin residues and melittin residues. Melittin is the main cytotoxic component of the *Apis mellifera* honeybee and is known to interact with lipid membranes. The hybrids demonstrated antimicrobial activity comparable to that of the parent compounds without the undesirable cytotoxicity of melittin to eukaryotic cells. Purified peptides of 18, 15, and 12 residues were compared with the antimicrobial effectiveness of 0.3% gentamicin and showed equal antimicrobial activity against both a clinical isolate and an ATCC strain of bacteria. This study confirmed in vivo the results of previous in vitro studies that demonstrated the broad antimicrobial spectrum of hybrid peptides.<sup>186</sup>

## EXPERIMENTAL DESIGN AND RATIONALE

In the present set of experiments, we endeavored to select an appropriate candidate peptide from a potential field of millions of peptides for testing against a panel of pathogens chosen for their frequency as causes of clinical keratitis in the United States. To achieve this selection, we turned to newly available computer technology for the design and choice of peptides. We elected to test these peptides in three settings: (1) in vitro, in a highly controlled system for performing quantitative microbiology, (2) in corneal storage media—a controlled system with direct relevance to corneal preservation and transplantation, and (3) in an in vivo animal model designed to assess both clinical outcomes of therapy and quantitative microbiological evaluation.

#### SELECTION OF A CANDIDATE PEPTIDE

In the vast majority of previous studies in which the rationale was the application of peptides for the testing of antimicrobial activity, researchers have worked with a candidate peptide primarily on the basis of its availability and its demonstrated in vitro spectrum of activity. The substances have either been extracted from cells or sequenced, synthesized and, in many cases, modified in order to make a synthetic peptide analogue that would theoretically demonstrate enhanced microbicidal activity. This methodology, employed by our laboratory as well as many others over the years, is limited by both availability and spectrum. That is, the candidate peptide chosen, one of potentially millions of candidates, may not be the optimal substance for the desired application and spectrum of activity. We concluded that random selection of peptides in this fashion would ultimately fail.

Therefore, for the purposes of the present research, we elected to determine the "best fit" candidate peptide according to a proprietary methodology for computational drug design devised by CyberChemics, Inc (Huntsville, Alabama). This approach is based on the use of powerful hardware- and software-based methods employing neural networks, artificial intelligence, and genetic algorithms for high-speed pattern recognition geared to identify the non-intuitive or hidden spatial pattern underlying the atoms that make one drug more effective than another. Representing a type of "computational genetic breeding," the methodology promotes marked acceleration of the screening process for potential candidate peptide selection by the medicinal chemist. The proprietary hardware is based on a parallel processor chip that allows the screening to be performed at supercomputer speeds. This technology adds to traditional in vitro and in vivo screening what CyberChemics, Inc, has termed "in vitro" screening—a computational candidate peptide selection that enhances the traditional screening process by eliminating millions of less effective conformational structures and limits the number of compounds that actually require synthesis and testing. Using such technology,  $10^{18}$  (a billion billion) small molecular building blocks can be scanned to produce a selection of the 100 most probable candidates for synthesis and testing. This novel method of combinatorial chemistry affords the ability to both diversify and select the most probable active sequences by using specially designed computer algorithms. In this way, extremely rapid identification, synthesis, and testing of therapeutic agents for infectious diseases can be achieved. Utilizing these methods, CyberChemics, Inc, has collected a library of over a million antimicrobial agents, a subset of which have been synthesized and tested, demonstrating inhibitory concentrations against significantly resistant infectious organisms.

The selection method uses a suite of pattern recognition algorithms that sort through viable substitutions in antimicrobial peptides. This search strategically selects substitutions that have occurred previously in molecular evolution (eg, in marine, amphibian, reptilian, mammalian, and avian peptides). The algorithm uses this substitution strategy to enhance the synthetic peptides with respect to their pharmacokinetics, bioavailability (predominantly hydrophilicity), resistance to proteases, and reduction of molecular weight.

CyberChemics, Inc, de novo peptides have been tested against *Escherichia coli*, *P aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *S aureus*, *S epidermidis*, *Aspergillus fumigatus*, and *C albicans*, among others. In addition, the technology has been applied to the development of peptide sequences that act as HIV-1 and hepatitis C virus protease inhibitors for the treatment of acquired immunodeficiency syndrome and hepatitis C.

For the present experiments, we used two CyberChemics, Inc, peptide sequences that were chosen on the basis of specification of organisms that are most commonly encountered in cases of human microbial keratitis in the United States. From a potential screening pool of 100 compounds, these compounds represented a cross section of sequence diversity with an available spectrum of biologic activity primarily against *Pseudomonas* species. The compounds were chosen with use of the algorithm according to behavior criteria, including bioavailability, potency, toxicity, and hydrophobicity. The activity of these peptides was reported by in 1998 (Noever D. Neural network for predicting ophthalmically relevant log P [octanol/water partition coefficients] for peptide antimicrobials. NCI Developmental Therapeutics Program. 1998 Western Multiconference, January 1998, San Diego, California).

Extensive evidence in the antimicrobial peptide literature demonstrates that the use of naturally occurring peptides (retrieved by extraction and purification) is both more expensive and less practical than the use of synthetically derived and optimized compounds. Both quantity and purity can be maximized by using synthetic techniques. There are currently no published data that directly compare the activity of the compounds used in this study with naturally occurring antimicrobial peptides.

The first group of these compounds was used in our in vitro microbial assay system as well as in corneal preservation media. Designated as CCI A, B, and C, the sequences of the compounds are as follows:

CCI A: LVLLKLLMKKYKLLKLLGGL  
CCI B: LLLLKLLLKNPKLKKLIGV  
CCI C: LLLLKLLKLLMNLLKLLGHY

The second compound is designated as COL-1, and the amino acid sequence is as follows:

COL-1: LVLLKKLMMKKYKLLKLLGGL

(Note: The amino acid key is as follows: A = alanine; C = cysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; N = asparagine; P = proline; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.)

#### SELECTION OF A PANEL OF PATHOGENS

Microbes used in this study were selected from a panel of human clinical ocular isolates from severe cases of bacterial keratitis managed in the Department of Ophthalmology, University of California, Davis, and maintained by the Microbiology Laboratory and by the Dairy Food and Safety Laboratory, School of Veterinary Medicine, University of California, Davis. The test panel of human clinical isolates maintained by our microbiology laboratory includes *P aeruginosa*, *M morgani*, *S pneumoniae*,  $\alpha$ -hemolytic *Streptococcus*, *S aureus*, *S epidermidis*, and *C albicans*.

The frequency of an organism as an ocular pathogen is modulated to some extent by local climate, the age of the population affected, and whether the country is a developing nation.<sup>30</sup> About 90% of cases of bacterial keratitis in the United States are caused by one of four groups of organisms: (1) *P aeruginosa*, (2) *S aureus* and *Micrococcaceae*, (3) *S pneumoniae*, and (4) *Enterobacteriaceae*.<sup>174</sup> *P aeruginosa* accounts for 8% to 23% of cases of bacterial keratitis in the United States, but this rate increases to 40% to 75% of the cases in contact lens wearers, while about 25% are due to *S aureus*, *S pneumoniae*, and other gram-positive cocci.<sup>175,176</sup>

*Pseudomonas* keratitis is one of the most serious corneal infections and represents one of the most threatening bacterial infections of the eye. Although it can be seen in specialized populations such as farmers and coal miners, the most common context is the population of contact lens wearers, where the infection results from an inoculum of microbes to the corneal surface in which the integrity of the epithelium has been breached. Because of its aggressive behavior and the frequency and context in which it occurs, *P aeruginosa* was chosen as the primary target pathogen in this study. *S aureus* was used as a test pathogen likewise because of its frequency as a clinical pathogen. *S epidermidis* is uncommonly a clinical corneal pathogen; however, its common presence at the ocular surface and its occasional conversion to an opportunist led to its selection as a comparison test organism. Because of the significant difficulties of managing the fastidious *S pneumoniae* in culture systems—a problem that makes bacterial quantification more difficult in the context of a quantitative, longitudinal study—we elected not to include

this organism in the panel.

#### DEVELOPMENT OF AN IN VIVO MODEL OF EXPERIMENTAL BACTERIAL KERATITIS

A number of animal models of microbial keratitis have been described in the past decade for reproducible evaluation of the pathogenesis of corneal infections and potential treatment regimens.<sup>177-192</sup> After experimentation with a number of different models, we developed a model that reliably produced a keratitis using conditions that simulate the clinical setting. This model combines a standardized epithelial defect, a single stromal crosshatch to ensure an interstice for the attachment of bacterial pathogens, and a standardized topical inoculum of bacteria. The procedure is described in detail in the “Materials and Methods” section under “In vivo experiments.”

#### MATERIALS AND METHODS

##### IN VITRO EXPERIMENTS

We tested the antimicrobial peptides COL-1 and CCI A,B, and C against the following organisms: *P aeruginosa*, *S aureus*, and *S epidermidis*. The organisms were human ocular (HO) isolates obtained from cases of severe human keratitis, cultured and stored by our microbiology laboratory. The assay employed is a quantitative microbial killing assay based on the principle that the precise amount of bacterial killing can be accurately determined only if one starts with a known quantity of organisms. This protocol allows one to obtain a stock solution of  $1 \times 10^6$  colony-forming units per milliliter (CFU/mL) in the incubation mixture.

##### Preparation of Bacteria in Log Phase of Growth

Twenty-four hours prior to the assay, we removed 2 to 5 well-isolated colonies of the desired organism from a blood agar plate and inoculated a bottle containing 40 mL of trypticase soy broth (TSB). This was incubated for 18 to 24 hours at 37°C. A 1-mL aliquot of the incubated TSB was then added to a bottle of sterile TSB and allowed to incubate in a shaking water bath at 37°C until the spectrophotometric optical density (OD) at 580 nm had increased tenfold, indicating that the bacteria were in log-phase growth (Figure 2).

The 40-mL suspension of bacteria was then centrifuged, washed with 10 mM sodium phosphate buffer (pH 7.4) twice, and resuspended in 10 to 15 mL of 10 mM sodium phosphate buffer (pH 7.4) (Figure 3).

The spectrophotometer (Beckman Du-50, Beckman Instruments, Fullerton, California) at 580 nm was used to verify a preparation of  $1 \times 10^7$  CFU/mL by comparison of the optical density to known standards.

##### Peptide Addition to Stock Solution

Ten microliters of this “stock” solution of bacteria was then

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added to the test tubes containing 80  $\mu\text{L}$  of 10mM sodium phosphate buffer (pH 7.4) and 10  $\mu\text{L}$  of the antimicrobial peptide (COL-1 in 0.5% methylcellulose and 0.05% EDTA) at a specified concentration (Figure 4).

In the test circumstance, therefore, the bacterial concentration was  $1 \times 10^6$  CFU/mL. The diluted solution was plated onto tryptic soy agar (TSA) plates at 0, 30, and 60

minutes. Each test was matched with a control tube that did not contain the peptide but did contain the solvent in which the peptide was dissolved. Control tubes contained 10  $\mu\text{L}$  of bacterial suspension, 80  $\mu\text{L}$  of 10 mM sodium phosphate buffer (pH 7.4), and 10  $\mu\text{L}$  of peptide solvent. A test tube and a control tube were prepared for incubation at each of three temperatures: 4°C, 23°C, and 37°C (ie,

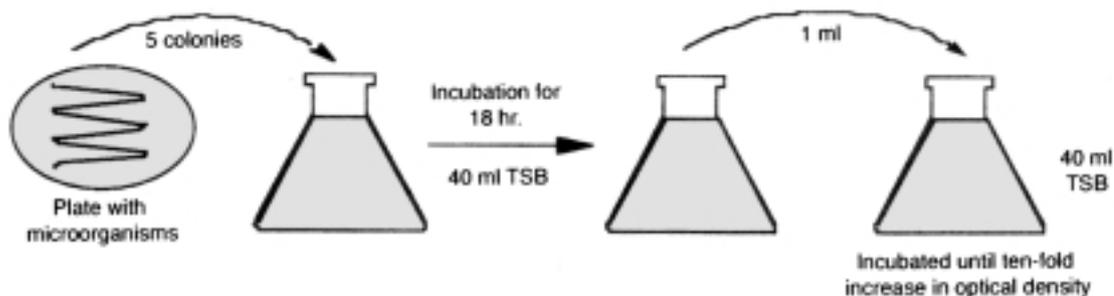


FIGURE 2

Preparation of a solution to achieve a suspension of bacteria in log-phase growth.

*Adapted from de Sousa.<sup>55</sup>*

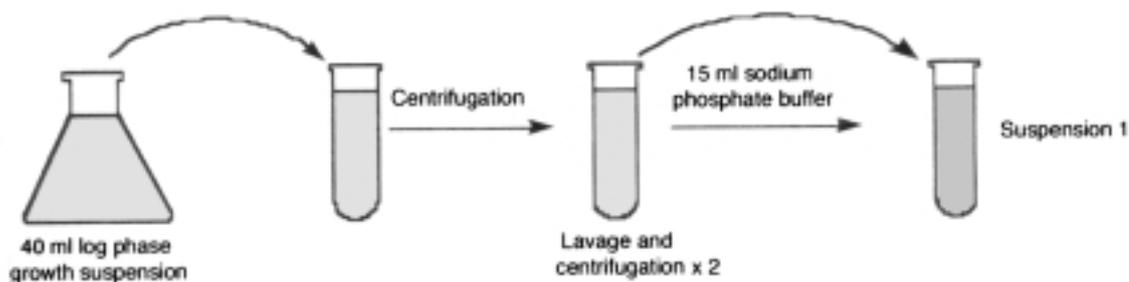


FIGURE 3

Centrifugation and resuspension of log-phase organisms prior to spectrophotometric density verification

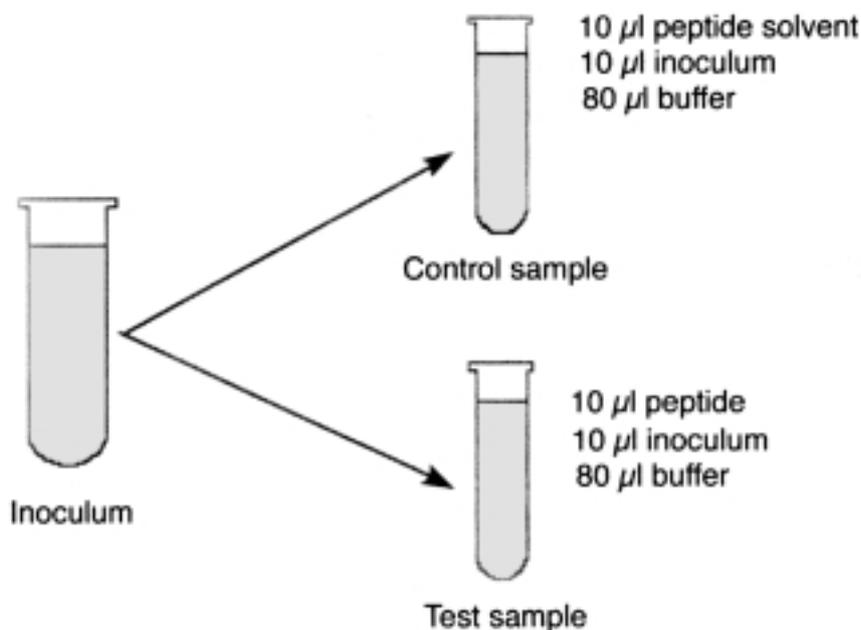


FIGURE 4

Preparation of control and test samples.

the temperature at which corneal storage media is maintained, room temperature, and standard incubation temperature).

#### Quantitative Microbial Assay

At specified time points, 10  $\mu\text{L}$  of test solution was removed and diluted 100-fold in 10 mM sodium phosphate buffer (pH 7.4). The dilution sample was plated in duplicate with a spiral plater (model D, Spiral System Instruments, Bethesda, Maryland) (Figure 5) onto TSA plates, which were incubated at 37°C for 18 to 20 hours, a period suitable for obtaining an adequate colony growth for quantitative analysis.

The spiral plater is a device that accurately distributes a liquid sample onto the surface of a rotating agar plate for the purposes of precise bacterial enumeration and antimicrobial susceptibility testing (Figure 6). A specific aliquot of solution (test or control) is drawn into the plater and deposited on the surface of a rotating agar plate. The volume deposited is controlled by a cam-activated syringe and decreases logarithmically with distance from the center of the plate. In this fashion, we were able to achieve a precise distribution that affords extremely accurate colony counts. Counts were completed automatically by the Synoptics Protos Plus Colony Counter (Microbiology International, Frederick, Maryland) (Figure 6). This device works by producing a video image of the agar plate and projecting this image onto a computer monitor. The colonies to be counted are then highlighted and counted automatically by the software program.

The instrument takes into account the dilution of the material plated and provides an accurate count of CFU/mL. At the conclusion of these procedures, we had determined a CFU/mL value for test and control solutions at 0, 30, and 60 minutes and at 4°C, 23°C, and 37°C for each concentration of peptide. From these values and our determination of the initial concentration, we used a formula ( $\log [N \text{ CFU of control}/N \text{ CFU of test}]$ ) to calculate the log reduction for each sample. (Note: With use of the spiral plater system and Protos colony counter, there is a theoretical lower limit of detection of  $1.02 \times 10^3$  CFU/mL.) Figure 7A demonstrates the three test-panel organisms after distribution by the spiral plater and incubation

Figure 7B demonstrates the comparative results of sample plates without exposure to the antimicrobial peptide (left) and with exposure to the antimicrobial peptide, COL-1 (right).

#### CORNEAL PRESERVATION MEDIA EXPERIMENTS

We tested the antimicrobial peptides CCI A, B, and C against the following organisms: *P aeruginosa*, *S aureus*, and *S epidermidis*. The methods were identical to those described previously with the following exceptions: (1) when a solution with a concentration of  $1 \times 10^6$  CFU/mL

was achieved, the stock solution was then added to test tubes containing 80  $\mu\text{L}$  of Optisol modified by the exclusion of antibiotics (Bausch & Lomb, Irvine, California) and 10  $\mu\text{L}$  (200  $\mu\text{g}/\text{mL}$ ) of the antimicrobial peptide (CCI A, B, or C); (2) experiments were performed at 4°C and 23°C only; and (3) exposure times were extended to include time points at 90 and 120 minutes, owing to the extended time that corneas are stored in preservation media. In addition, the experiments were repeated using the test organism (10  $\mu\text{L}$ ), Optisol (70  $\mu\text{L}$ ) with 100 mM EDTA (10  $\mu\text{L}$ ), and peptide (10  $\mu\text{L}$ ).

#### IN VIVO EXPERIMENTS

This phase of the project was designed to determine the antimicrobial efficacy of COL-1 when applied topically to an experimentally induced *P aeruginosa* keratitis. (All experimental animals were managed and cared for under approved institutional review board guidelines by the Animal Resources Department at the University of California, Davis. These guidelines adhered to the principles for animal experimentation of the Association for Research in Vision and Ophthalmology.)

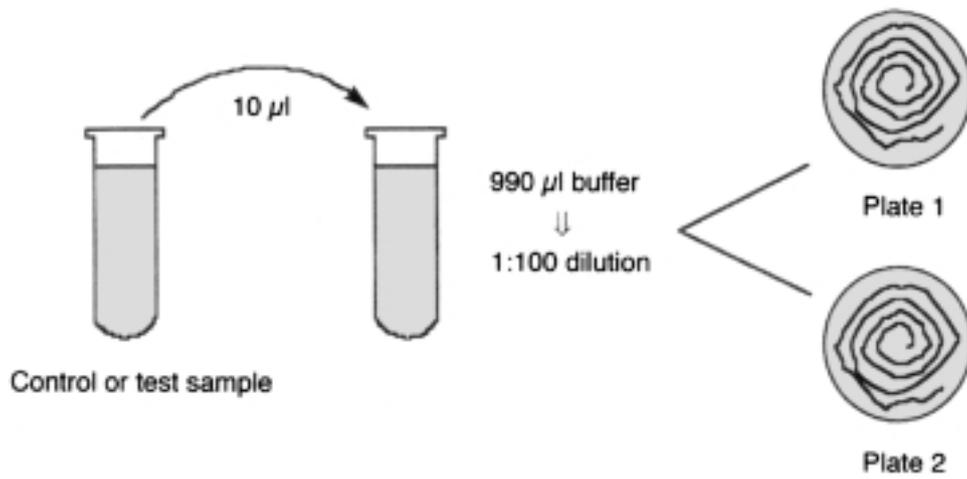
#### Preparation of *Pseudomonas aeruginosa*

Two to 5 colonies of *P aeruginosa* (HO-31) were selected from a pure culture plate for inoculation into 40 mL of TSB. This culture was incubated for 18 hours at 37°C, yielding approximately  $1 \times 10^9$  CFU/mL as determined by previous counts. Ten microliters of the overnight culture was diluted into 990  $\mu\text{L}$  of 10 mM sodium phosphate buffer (pH 7.4) to achieve a concentration of  $\approx 1 \times 10^7$  CFU/mL. A Hamilton microliter syringe was used to deliver two 10- $\mu\text{L}$  aliquots of this "stock" solution to the cornea, resulting in an approximate delivery to the cornea of  $2 \times 10^5$  total CFU.

#### Method of Inoculation

Each rabbit received a subcutaneous injection consisting of a mixture of xylazine hydrochloride (15 mg) and ketamine hydrochloride (125 mg). Once the rabbit was sedated, we placed 2 to 3 drops of proparacaine hydrochloride ophthalmic solution (USP 0.5%) on the right eye of the animal, and a lid speculum was introduced. Under observation with the operating microscope, a 6.5-mm trephine was used to demarcate a central area for de-epithelialization. The epithelial layer of cells was removed with a No. 15 surgical blade, exposing the stroma. A superficial crosshatch ("X") was scored into the anterior stroma with a 22-gauge needle. Once the site for inoculation was prepared, two 10- $\mu\text{L}$  aliquots of the stock solution were dropped onto the prepared surface with the Hamilton syringe (20 to 30 seconds between doses).

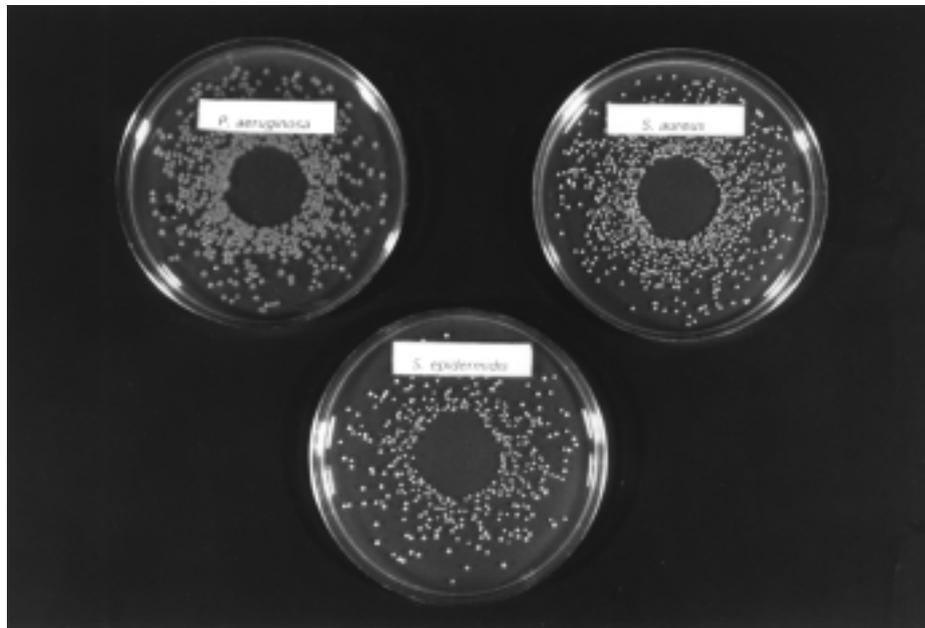
*The Use Of Antimicrobial Peptides In Ophthalmology*



**FIGURE 5**  
Diluted samples are placed on solid agar using the spiral plater.

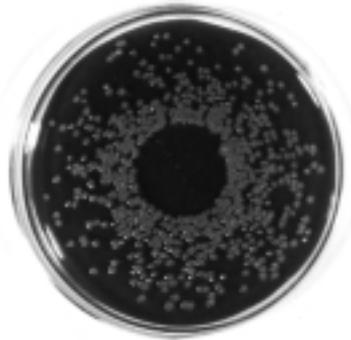


**FIGURE 6**  
Spiral plater system (left). Protos plate reader (right).



**FIGURE 7A**  
The 3 test organisms at T=0.

*P. aeruginosa*

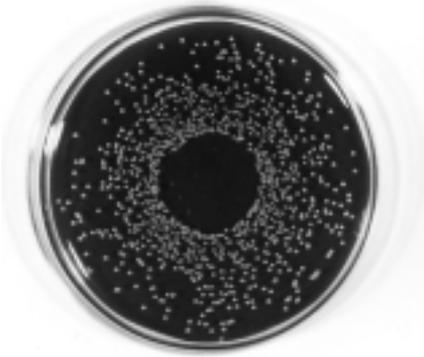


Control



Test

*S. aureus*

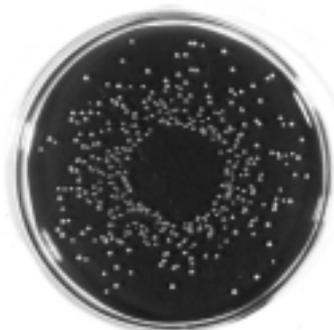


Control



Test

*S. epidermidis*



Control



Test

FIGURE 7B

Control and test plates demonstrating the activity of peptide COL-1 versus the three test organisms in this study: *P. aeruginosa* (top), *S. aureus* (middle), and *S. epidermidis* (bottom).

### Treatment Schedule

The inoculum was allowed to incubate for 12 to 14 hours prior to initiation of treatment. The treatment schedule was as follows (except as noted):

- Day 1: One drop (containing either a specified concentration of COL-1, 10 or 50 µg/mL; tobramycin 0.3%; or 0.5% methylcellulose and .05% EDTA) every 15 minutes for the first hour, followed by 1 drop every hour for the next 9 hours. Dosage amounts of either 10 µg/mL or 50 µg/mL were chosen, since they represented the lowest effective in vitro dose and the highest dose that was not toxic to test animals in the toxicity trials.
- Days 2 through 4: One drop every hour for 10 hours. (Note: For the initial in vivo experiments, the treatment schedule differed slightly. Infection was allowed to incubate for 24 hours before treatment was initiated. The treatment schedule for days 1 through 3 was 1 drop every hour for 10 hours, and for days 4 through 6, 1 drop four times a day, 8 AM to 5 PM.)

### Observation

During the treatments, conjunctival hyperemia, discharge, corneal opacification and suppuration, and general behavioral responses were observed and recorded.

### Euthanization

At the conclusion of the test period, each rabbit received a mixture of 15 mg xylazine hydrochloride and 125 mg ketamine hydrochloride subcutaneously. Once anesthetized, each rabbit received an intracardiac injection of sodium pentobarbitol (390 mg/mL).

### Microbiological Analysis

A corneal-scleral button was excised from the right globe, and an 8-mm button was punched on a Teflon dish and placed into 2 mL of 10 mM sodium phosphate buffer (pH 7.4). The tissue was then homogenized using a Powergen 125 (Fisher Scientific) tissue homogenizer. The homogenate was centrifuged at 700g (1,500 rpm) for 7 minutes and the supernatant removed. Three 1:100 serial dilutions of the supernatant were made in 10 mM sodium phosphate buffer (pH 7.4), and each dilution plus an undiluted sample of the supernatant was plated on TSA plates in duplicate using the spiral plater. The plates were incubated for 18 to 20 hours at 37°C.

### EX VIVO TOXICITY STUDIES

Because certain animals demonstrated inflammation, which the investigators thought was related directly to the peptide instillation, we performed ex vivo toxicity studies on the corneal endothelium using sheep corneas and a range of peptide concentrations.

Whole sheep globes were obtained from freshly slaughtered animals (Superior Farms, Dixon, California). Testing procedures were performed within 2 to 4 hours after harvesting of the globes. The corneas were excised using corneal-scleral scissors with care taken not to contact the corneal endothelium or to fold or compress the tissue. Corneal scleral buttons were then placed epithelial side down in a Teflon dish and exposed to COL-1 at concentrations of 25, 50, and 100 µg/mL for 15 seconds or 60 seconds. Control corneas were exposed to carrier substances alone, including phosphate buffered saline (PBS), pH 7.4; 0.5% methylcellulose and 0.05% EDTA; and 10 mM sodium phosphate buffer (pH 7.4). Immediately after exposure, corneas were gently rinsed in PBS and were stained using a vital staining technique with trypan blue and alizarin red. Vital staining was performed by exposing endothelium to 0.25% trypan blue for 90 seconds followed by a gentle rinse in PBS, after which alizarin red was applied for 45 seconds. Corneas were immediately examined under a light microscope equipped with a standardized grid for cell counting. Two grid blocks (~500 cells) placed over the central cornea were counted for each specimen, and an average count was determined. Between 3 and 6 repetitions were performed at each concentration of peptide. The results were expressed as a percentage of cells staining with trypan blue as an index of those cells with abnormal permeability.

## RESULTS

### IN VITRO EXPERIMENTS

#### COL-1 Versus Human Ocular Isolates

Results are expressed as log reduction in CFU/mL. Table V clarifies the relationship between log reduction and percent reduction of organisms. For example, a 3-log reduction represents eradication of 99.9% of bacterial growth.

Figures 8A, 8B, and 8C demonstrate the dose-response curves over time for *P aeruginosa* (HO-31), *S aureus* (HO-27), and *S epidermidis* (HO-29) at 37°C, 23°C, and 4°C, respectively. Each curve demonstrates reduction at times 0, 30, and 60 minutes for exposure to COL-1 at concentrations of 0.1, 1, 10, 25, and 50 µg/mL in 0.5% methylcellulose and 0.05% EDTA. Peptide in solution was added to 10 mM sodium phosphate buffer in which the microbial assay was performed. Given our

TABLE V: CORRELATION BETWEEN LOG REDUCTION AND PERCENT REDUCTION OF ORGANISMS

LOG REDUCTION	% REDUCTION
1-log	90%
2-log	99%
3-log	99.9%
4-log	99.99%

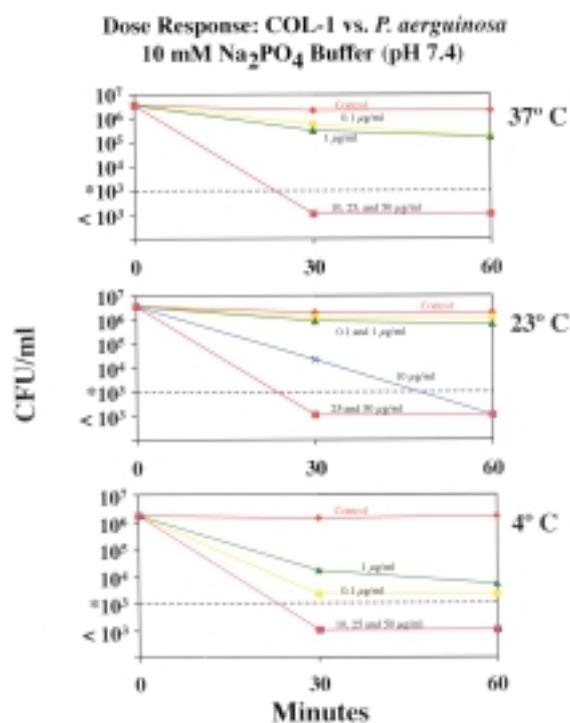


FIGURE 8A

Dose-response curve for COL-1 versus *P. aeruginosa* at 37°C, 23°C, and 4°C. Dashed line represents lower limit of detection for assay conditions. (Each curve is a representative graph of an experiment that was performed in triplicate.)

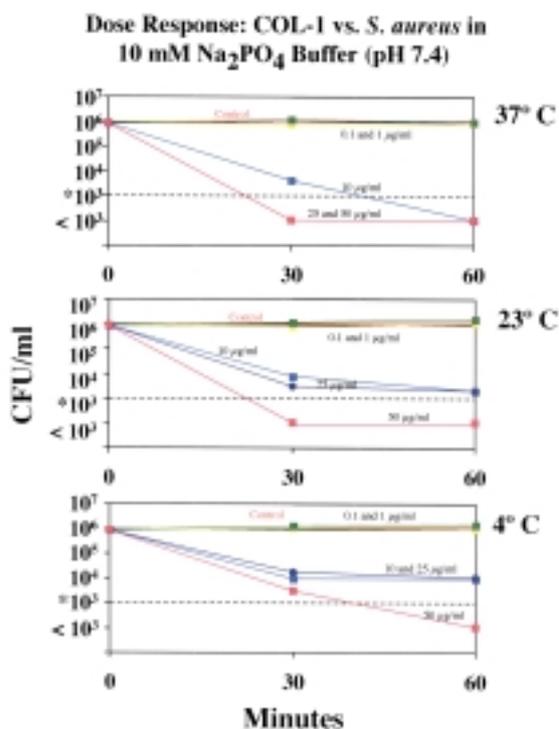


FIGURE 8B

Dose-response curve for COL-1 versus *S. aureus* at 37°C, 23°C, and 4°C. Dashed line represents lower limit of detection for assay conditions. (Each curve is a representative graph of an experiment that was performed in triplicate.)

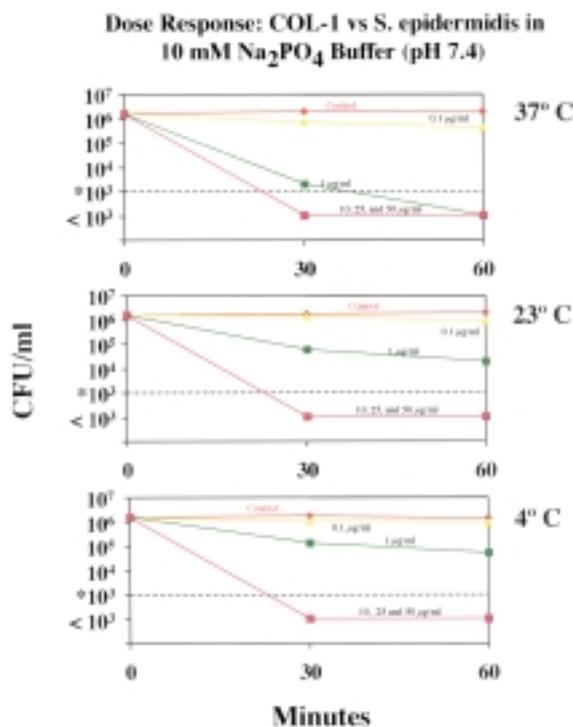


FIGURE 8C

Dose-response curve for COL-1 versus *S. epidermidis* at 37°C, 23°C, and 4°C. Dashed line represents lower limit of detection for assay conditions. (Each curve is a representative graph of an experiment that was performed in triplicate.)

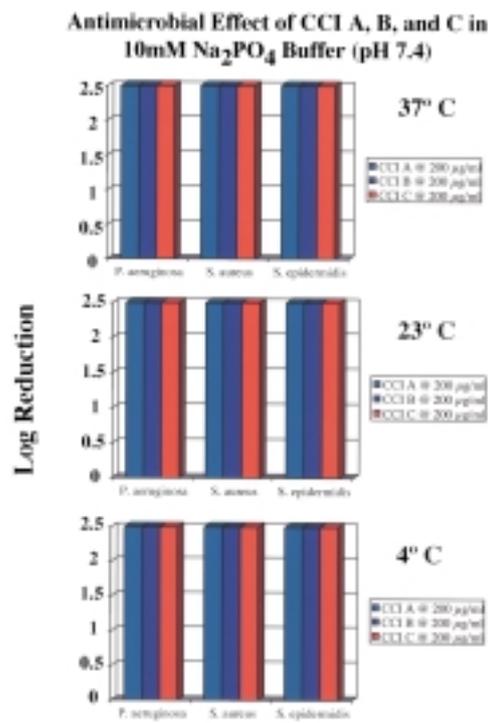


FIGURE 9

Log reduction induced by CCI A, B, and C against 3 test isolates at 37°C, 23°C, and 4°C in phosphate buffer.

lower limit of detection in this assay of  $1.02 \times 10^3$  CFU/mL, points denoted as  $<10^3$  CFU/mL represent plates with no colonies.

As indicated in Figures 8A, 8B, and 8C, for *P aeruginosa* and *S epidermidis*, there was greater than a 3-log reduction at 25 and 50  $\mu\text{g/mL}$  COL-1 at all temperatures tested. For *S aureus*, there was complete eradication with 50  $\mu\text{g/mL}$  at all temperatures. However, at 23°C, log reduction was less than 2.5 at 25  $\mu\text{g/mL}$  COL-1 but complete at 50  $\mu\text{g/mL}$ . At 4°C, complete reduction was not obtained against *S aureus*, but log reduction was 2.50 (>99% reduction). Although there is a significant decrease in organism count at lower peptide concentrations, there is, in general, a distinct fall-off of peptide activity between 10 and 25  $\mu\text{g/mL}$ , and at these lower levels, a total reduction is not obtained. The exceptions to this were *S epidermidis* at 37°C, where total eradication was obtained down to 1  $\mu\text{g/mL}$ , and *P aeruginosa* at 4°C, where total eradication was obtained down to 10  $\mu\text{g/mL}$ .

*CCIA, B, and C Versus Human Ocular Isolates (in 10 mM sodium phosphate buffer, pH 7.4)*

Figure 9 demonstrates the log reduction of CFU for *P aeruginosa* (HO-31), *S aureus* (HO-27), and *S epidermidis* (HO-29) at 37°C, 23°C, and 4°C, respectively. Results are expressed as log reduction (log killing) in CFU/mL. Each graph demonstrates killing of each organism using the three peptides CCI A, B, and C at concentrations of 200  $\mu\text{g/mL}$  in 10 mM sodium phosphate

buffer (pH 7.4). Peptide in solution was added to 10 mM sodium phosphate buffer in which the microbial assay was performed. Given our lower limit of detection in this assay, complete killing is represented as  $1.02 \times 10^3$  CFU/mL. Log reduction of 2.5 or more represents no colonies on the plates in this assay.

The peptide was effective in producing complete killing at this concentration at all three temperatures.

*CCIA, B, C in Modified Optisol*

Figure 10 demonstrates log reduction of bacterial CFU in modified Optisol (without antibiotics) at 4°C and 23°C employing CCI A, B, and C at 200  $\mu\text{g/mL}$  against three human ocular isolates.

These data demonstrate that *S epidermidis* was effectively reduced by each of the CCI compounds at 4°C, but that neither *S aureus* nor *P aeruginosa* was affected significantly by the peptide in modified Optisol. At 23°C, peptide antimicrobial activity was augmented against the gram-positive organisms but had no effect on *P aeruginosa*.

The experiment was repeated at both 4°C and 23°C in Optisol with the addition of 100 mM EDTA in order to determine if peptide activity could be augmented by this addition. Previous experiments have demonstrated that EDTA, which chelates calcium and other divalent ions, augments killing by destabilizing gram-negative bacterial cell membranes. Virtually all ophthalmic preparations are formulated with 0.05% to 0.1% EDTA as a preservative.<sup>193-195</sup>

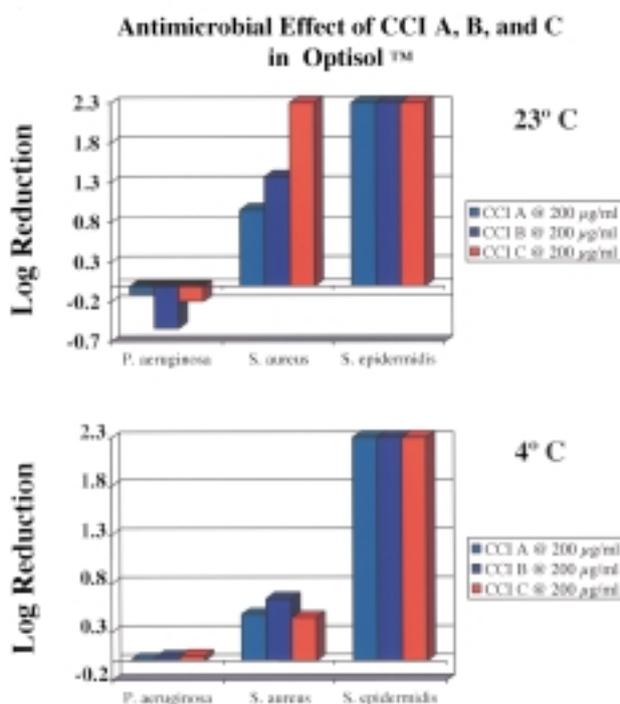


FIGURE 10

Log reduction induced by CCI A, B, and C against three test isolates at 23°C and 4°C in modified Optisol.

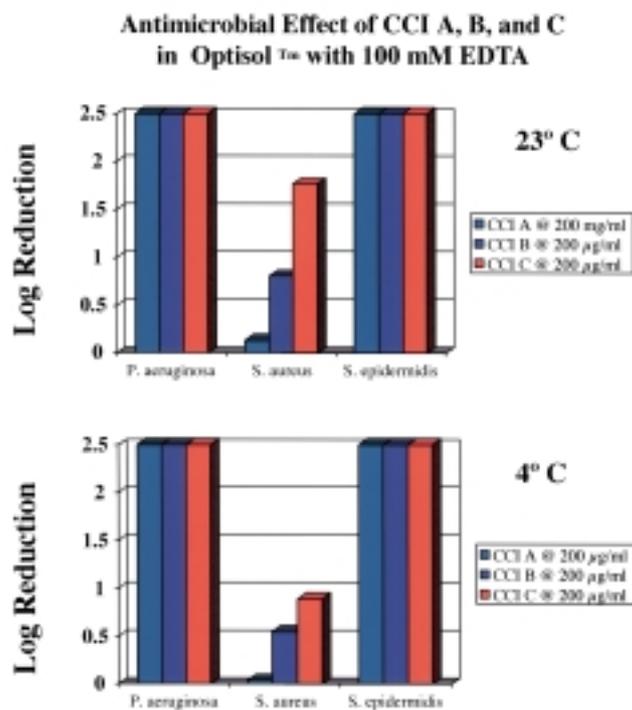


FIGURE 11

Log reduction induced by CCI A, B, and C against three test isolates at 23°C and 4°C in modified Optisol and EDTA.

### CCI A, B, C in Modified Optisol and EDTA

Figure 11 demonstrates the total log reduction of the same ocular isolates in modified Optisol and 100 mM EDTA at 4°C and 23°C. The data indicate that EDTA greatly augmented log reduction for *P aeruginosa* but made no difference for the gram-positive organisms.

These data demonstrate that the addition of 100 mM EDTA to modified Optisol produced complete killing of *P aeruginosa* at 4°C and 23°C but did not effectively augment killing of *S aureus* at either temperature.

### IN VIVO EXPERIMENTS

To demonstrate the effectiveness of topical peptides in a reproducible model of bacterial keratitis, a total of 59 rabbits in a series of different experiments were employed to test COL-1 against induced *Pseudomonas* corneal infection.

Table VI demonstrates that COL-1 was not effective in either the clinical outcome or the quantitative microbial analysis when used in the in vivo model ( $P = .19-.51$ ). Since the two groups we compared were not independent and normally distributed populations, we could not use a standard *t* test for comparison; we therefore employed a non-parametric test (Wilcoxon rank sum test using the SAS/STAT program).

Because in the previous experiment there was no difference between test and control rabbits, we performed an experiment to demonstrate the growth curve of human ocular pathogens in our rabbit keratitis model. The purpose was to determine if we were missing an effect on account of natural attrition of the bacteria in the host cornea over time. We generated a longitudinal growth curve by inoculating rabbit cornea with a total of  $3.84 \times 10^5$  CFU. At 14 hours, two untreated rabbits were sacrificed to obtain a pretreatment CFU count at the end of the incubation period. Subsequently, at 24, 48, 72, and 96 hours, we euthanized two control (given only 0.5% methylcellulose and 0.05% EDTA) and 2 test rabbits (given 50 µg/mL COL-1 in 0.5% methylcellulose and 0.05% EDTA). Figure 12 demonstrates the growth curves of the

Longitudinal Growth Curve of  
*P. aeruginosa* In Vivo

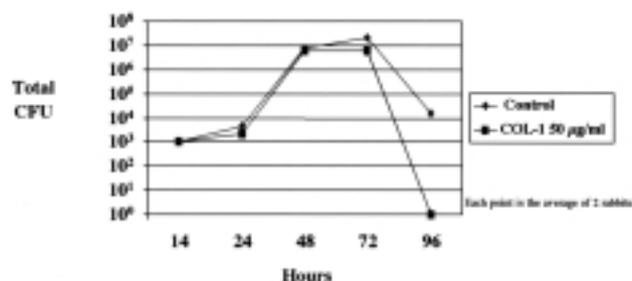


FIGURE 12

Longitudinal in vivo growth curves of *P aeruginosa* in test and control animals over 96 hours.

*P aeruginosa* in both test and control animals.

In either the treated or the untreated eye, bacterial counts begin to diminish naturally between 72 and 96 hours. This curve was generated so that this phenomenon of natural attrition could be separated from peptide effect, allowing us to interpret our results more accurately.

Table VII shows the results of treatment with 50 µg/mL of COL-1 in 0.5% methylcellulose and 0.05% EDTA compared with controls utilizing the methylcellulose carrier only and a control utilizing tobramycin 0.3%. All rabbits received an initial inoculum of  $4.05 \times 10^5$  CFU. The table shows that the tobramycin control was effective in eliminating both the clinical manifestations of the induced keratitis and the growth of bacteria. The activity of COL-1 showed no significant difference from the methylcellulose control in the quantitative assay ( $P = .33$ ). Clinically, the COL-1 test rabbits appeared to have more inflammation than the methylcellulose controls.

Table VIII presents the results of treatment with 50 µg/mL of COL-1 in 0.5% methylcellulose and 0.05% EDTA compared with controls utilizing the methylcellulose carrier only and a control utilizing tobramycin 0.3%. All rabbits received an initial inoculum of  $6.30 \times 10^5$  CFU.

This experiment demonstrates that the peptide was of

TABLE VI: COL-1 10 µG/ML EMPLOYED AGAINST PSEUDOMONAS KERATITIS (INOCULUM= $2.6 \times 10^5$  CFU/ML)

RABBIT	TEST/TREATMENT	CFU RECOVERED	OBSERVATIONS
1	COL-1 10 µg/mL*	150	Dense central corneal abscess
2	COL-1 10 µg/mL*	30	Diffuse conjunctiva, mildly dense central corneal infiltrate
3	COL-1 10 µg/mL†	100	Dense central infiltrate, pan corneal abscess
4	COL-1 10 µg/mL†	0	Mild central corneal infiltrate
5	0.5% Methylcellulose	100	Diffuse central abscess, dense paracentral infiltrate
6	0.5% Methylcellulose	10	Diffuse patchy infiltrate of the cornea
7	0.5% Methylcellulose plus 0.05% EDTA	150	Dense central corneal infiltrate
8	0.5% Methylcellulose plus 0.05% EDTA	170	Dense central corneal abscess

\* Peptide in 0.5% methylcellulose.

† Peptide in 0.5% methylcellulose plus 0.05% EDTA.

*The Use Of Antimicrobial Peptides In Ophthalmology*

**TABLE VII: RESULTS OF TREATMENT WITH 50 µG/ML OF COL-1 IN 0.5% METHYLCELLULOSE + 0.05% EDTA COMPARED WITH CONTROLS UTILIZING THE METHYLCELLULOSE CARRIER ONLY AND CONTROLS UTILIZING TOBRAMYCIN 0.3%**

RABBIT	TEST/TREATMENT	CFU RECOVERED	OBSERVATIONS
9	COL-1 50 µg/mL*	20	Small abrasion
10	COL-1 50 µg/mL*	1,070	Perforated, dense infiltrate
11	COL-1 50 µg/mL*	5,580	Perforated, dense infiltrate
12	COL-1 50 µg/mL*	874	Perforated, dense infiltrate
13	COL-1 50 µg/mL*	120	Perforated, dense infiltrate
14	COL-1 50 µg/mL*	14,100	Perforated, dense infiltrate
15	COL-1 50 µg/mL*	2,380	Perforated, dense infiltrate
16	COL-1 50 µg/mL*	3,660	Perforated, dense infiltrate
17	COL-1 50 µg/mL*	17,200	Perforated, dense infiltrate
18	Tobramycin	0	Small abrasion
19	Tobramycin	0	Small abrasion
20	Tobramycin	0	Small abrasion
21	Control†	0	Clear
22	Control†	5,020	Dense infiltrate
23	Control†	0	"X" score slightly visible
24	Control†	6,300	Perforated, dense infiltrate
25	Control†	40	Pin size abrasion
26	Control†	9,680	Dense infiltrate
27	Control†	4,920	Mild infiltrate
28	Control†	488	Perforated, dense infiltrate
29	Control†	0	2 pin size spots, mild injection

\* Peptide in 0.5% methylcellulose plus 0.05% EDTA.

† Control in 0.5% methylcellulose plus 0.05% EDTA.

**TABLE VIII: RESULTS OF TREATMENT WITH 50 µG/ML OF COL-1 IN 0.5% METHYLCELLULOSE AND 0.05% EDTA (BUFFERED) COMPARED WITH CONTROLS UTILIZING THE METHYLCELLULOSE CARRIER ONLY AND A CONTROL UTILIZING TOBRAMYCIN 0.3%**

RABBIT	TEST/TREATMENT	CFU RECOVERED	OBSERVATIONS
30	COL-1 50 µg/mL*	0	Quiet, small abrasion
31	COL-1 50 µg/mL*	7,160	Injected, dense infiltrate
32	COL-1 50 µg/mL*	0	Injected, dense infiltrate
33	COL-1 50 µg/mL*	40	Injected, dense infiltrate
34	COL-1 50 µg/mL*	12,000	Dense infiltrate
35	Tobramycin	0	Quiet, "X" score slightly visible
36	Tobramycin	0	"X" score slightly visible
37	Control†	0	Quiet
38	Control†	23,400	Injected, dense infiltrate
39	Control†	0	Small abrasion
40	Control†	0	Small abrasion
41	Control†	0	Quiet, pin size abrasion

\* Peptide in 0.5% methylcellulose plus 0.05% EDTA. (buffered)

† Control in 0.5% methylcellulose plus 0.05% EDTA. (buffered)

no advantage in the management of bacterial keratitis either clinically or microbiologically ( $P = .50$ ) and that, in fact, clinically, rabbits treated with the peptide demonstrated more inflammation than controls.

**TOXICITY STUDY**

Table IX demonstrates the results of the in vivo toxicity testing in which 1 drop of COL-1 (range, 10 to 3,000 µg/mL) was placed on the eye of a test rabbit every hour for 10 hours daily over a period of 4 days. Toxicity was

defined as diffuse conjunctival hyperemia. The trial was ended in any animal if hyperemia and edema were still present 24 hours after first initiating the medication.

Table X summarizes the results of the ex vivo endothelial toxicity studies in sheep corneas as indicated by vital staining of cells with trypan blue and alizarin red. We counted the percentage of cells demonstrating uptake of trypan blue as an indicator of cell wall damage.

These data demonstrate that COL-1 was toxic when directly applied to the corneal endothelium.

TABLE IX: RESULTS OF IN VIVO TOXICITY TESTING FOR A PREPARATION OF TOPICAL COL-1 (RANGE, 10-3,000  $\mu\text{G}/\text{ML}$ )

CONCENTRATION OF COL-1	TOXIC/NONTOXIC
10 $\mu\text{g}/\text{mL}$	Nontoxic
50 $\mu\text{g}/\text{mL}$	Nontoxic
100 $\mu\text{g}/\text{mL}$	Toxic
200 $\mu\text{g}/\text{mL}$	Toxic
380 $\mu\text{g}/\text{mL}$	Toxic
1,500 $\mu\text{g}/\text{mL}$	Toxic
3,000 $\mu\text{g}/\text{mL}$	Toxic

TABLE X: RESULTS OF EX VIVO ENDOTHELIAL TOXICITY STUDIES IN SHEEP CORNEAS

TREATMENT	TIME	AVERAGE % DAMAGE
COL-1 100 $\mu\text{g}/\text{mL}$	1 minute	37.4*
COL-1 50 $\mu\text{g}/\text{mL}$	1 minute	26
COL-1 50 $\mu\text{g}/\text{mL}$	15 seconds	21
COL-1 25 $\mu\text{g}/\text{mL}$	1 minute	16
PBS	1 minute	2.7
0.5% Methylcellulose + 0.05% EDTA	1 minute	2.5
10 mM $\text{Na}_2\text{PO}_4$ buffer (pH 7.4)	1 minute	7.9*

\* Because supply of corneas was limited, only one test was done for treatments: COL-1 100  $\mu\text{g}/\text{mL}$  and 10 mM  $\text{Na}_2\text{PO}_4$  buffer (pH 7.4).

## DISCUSSION AND DATA ANALYSIS

The work outlined in this thesis is directed to the discovery and development of synthetic pore-forming antimicrobial peptides for use in the treatment of microbial keratitis and is a novel method of preventing contamination in preservation systems for corneal transplantation.

Bacterial keratitis is a cause of significant morbidity worldwide and can cause rapid and devastating visual loss.<sup>196</sup> While bacterial keratitis may be associated with poor hygienic conditions and endemic diseases such as trachoma in some parts of the world, in the United States it is linked in large part to contact lens wear. In the 29 million contact lens wearers in the United States, the incidence of bacterial keratitis is about 1 in 1,000. Typically, such keratitis is rapid in onset and may be very destructive to the host cornea and thus to visual function. An example is *Pseudomonas* keratitis, in which much of the early damage to the cornea is the result of proteases generated by the bacteria. In the case of this pathogen, which represents up to 75% of contact lens-associated bacterial keratitis, delay in effective therapy becomes an even more pressing issue. Delay in both diagnosis and initiation of effective treatment could potentially blind an eye from early suppurative proteolytic destruction of the corneal structure.

Bacterial keratitis is generally treated intensively (every 15 to 60 minutes for several days) with combinations of topical fortified antibiotics such as cefazolin and tobramycin or gentamicin.<sup>197</sup> Recently, monotherapy with a topical fluoroquinolone has become a first-line approach in selected cases, while vancomycin is generally reserved for severe vision-threatening keratitis that is not responsive to first-line agents. The antimicrobials currently in use are problematic because of their toxic effects on the ocular surface (eg, punctate keratitis, delayed re-epithelialization, hyperemia, chemosis) and, more important, the emerging and increasing patterns of resistance. Aminoglycoside resistance in cases of keratitis, endophthalmitis, and infection of corneal donor material is on the increase.<sup>198,199</sup> While the fluoroquinolones have provided a reasonable tool,<sup>200</sup> there are early, disturbing reports of resistance to these agents as well.<sup>11-25</sup> With the advent of resistance to vancomycin, there is a more pressing need to find new antimicrobial alternatives that will affect a suitable spectrum of ocular pathogens and that will not be plagued by rapidly developing resistance. To date, reports of resistance to the antimicrobial peptides have been minimal, and because of their mechanism of action, bacterial resistance to peptides is likely to develop very slowly. There are only isolated reports of resistance to peptides in the microbiological literature; some bacteria are naturally resistant (eg, *Serratia marcescens*, *Burkholderia cepacia*) by virtue of a noninteractive outer membrane or the elaboration of specific proteases.<sup>2</sup> In addition, there are isolated reports of *Salmonella* resistance.<sup>201</sup>

The problem of infection after corneal transplantation is far less common than bacterial keratitis. Of the approximately 40,000 transplants performed in the United States each year, the incidence of endophthalmitis is 0.1% (1998 Statistical Report, Eye Bank Association of America, Washington, DC). The precise incidence of postkeratoplasty keratitis is not as easily pinpointed. Nonetheless, the occurrence of keratitis or endophthalmitis after keratoplasty can represent a devastating complication and can be related to a variety of risk factors, including contamination of donor tissue, intrasurgical inoculation, and postoperative infection.<sup>202</sup> In the event of an infection following keratoplasty, early recognition of the pathogen becomes crucial for successful treatment. A variety of organisms have been implicated, including *S epidermidis*, *S aureus*, *S pneumoniae*, *Streptococcus viridans*, *P aeruginosa*, *S marcescens*, *Haemophilus influenzae*, *Bacillus* species, and *C albicans*.<sup>203-213</sup>

To diminish the risk of contamination of corneal storage media, a variety of antibiotics, antiseptics, and peptides have been investigated as additives to preservation media with emphasis on finding a nontoxic agent effective at standard storage temperatures.<sup>164,214-223</sup> Among the

antimicrobials investigated in preservation medium are penicillin, streptomycin,<sup>210,224</sup> gentamicin,<sup>210,214,225</sup> cefazolin,<sup>215</sup> povidone iodine,<sup>219</sup> and vancomycin.<sup>226</sup> Currently, gentamicin plus streptomycin is the most commonly used combination in corneal storage media, although gentamicin alone may not affect the *Streptococcus* species, *S epidermidis*, and *S aureus*, the species most commonly isolated from corneal scleral rims at the time of tissue harvest.<sup>212,227-230</sup> The special circumstance of corneal storage at 4°C and the closed system provide a challenge for adequate effective sterilization of the media without damage to the delicate corneal tissue. Although Hwang and colleagues<sup>221</sup> and Lass and colleagues<sup>223</sup> demonstrated that the antimicrobial activity of the antibiotics in preservation media can be augmented by leaving the media containing the tissue at room temperature for about 1 hour prior to preservation at 4°C, it would be ideal to employ an agent that is effective at all storage temperatures. The ideal antimicrobial agent has not been found, especially considering these storage conditions and the lengthier storage times for shipment of tissue globally for transplantation. The resistance data stress the need for an antimicrobial agent that is effective at all temperatures and that covers the common contaminants.

#### DATA ANALYSIS

##### *Interpretation of Our Data in Context*

The data selected for presentation in this series reflect a distillation of recent efforts in our laboratory to demonstrate a promising candidate peptide for application to ocular infection. We attempted to utilize a new methodology of candidate peptide selection with the greatest potential for success for use as a topical antimicrobial agent, and we employed these peptides in two in vitro situations—in a standard controlled 10 mM sodium phosphate buffer for microbiological analysis and in modified corneal preservation media. Finally, we established a working in vivo animal assay in which to test the peptide as a topical application. Our data demonstrate that although the peptides used in this study were effective against serious corneal clinical isolates in a closed in vitro system, the peptides were not effective against all bacteria tested in either corneal preservation media or at the ocular surface in an animal model.

The results demonstrate that in a highly controlled phosphate buffered saline system in which electrolytic composition and pH are rigidly controlled, the candidate peptides proved effective against the clinical isolates we tested. In a similarly controlled but very different system—that of chondroitin sulfate-based corneal storage media—the results were less impressive. And in a reliable in vivo animal model, the candidate peptides demonstrated no effect either clinically or microbiologically. The

outcome of this study will be of value for what we have learned about the selection and application of these substances in the testing for ophthalmic applications.

In summary of the experimental results, we can conclude the following:

1. The candidate peptides (CCI A, B, and C and COL-1) were successful in producing a total kill of the test clinical isolates in 10 mM sodium phosphate buffer.
2. In modified Optisol, the peptides were effective against *S epidermidis* at all temperatures, demonstrated augmented activity at 23°C against the gram-positive organisms, but were ineffective against *P aeruginosa*. The addition of EDTA to the medium augmented the killing of *P aeruginosa* but made no difference in the reduction of gram-positive organisms.
3. In an in vivo rabbit model of *Pseudomonas* keratitis, COL-1 demonstrated neither clinical nor microbicidal efficacy and appeared to have a very narrow dosage range, outside of which it appeared to be toxic to the ocular surface.

Although the peptides selected showed substantial microbicidal activity in the initial postformulation testing stage, we must try to understand why they were unsuccessful in corneal storage media and at the ocular surface, both of which represent important clinical circumstances in which infection may be a serious issue.

In the important circumstance of modified corneal storage media, there are several possibilities for inhibition of the microbicidal action. It has been well established that ionic shifts and alterations in pH affect the lytic activity of peptides.<sup>101,154,231</sup> Indeed, their capability as pore-forming agents depends on electrostatic charge distribution and their interaction with the target-cell membrane. It is possible that the chondroitin sulfate or the ionic strength of Optisol alters or masks the electrostatic distribution of the peptide. Chondroitin sulfate has 2 negative charges on its upper face, which, in contact with the peptide, could block the positive charges that are so crucial to its mechanism of action.<sup>55,232</sup> The same problem was not exhibited by defensins in previous reports with the chondroitin sulfate-based Optisol, possibly because the defensin molecule is more globular and irregular and does not expose a long positively charged face to the chondroitin sulfate polymer.<sup>218,232</sup> Other components found in Optisol, such as calcium, dextran, and Hepes buffer, could also affect peptide activity.

The fact that we were able to demonstrate some activity of the peptide in modified Optisol, especially with the addition of EDTA, suggests to us that there may be ways of altering the composition that would favor the activity of the peptide in the medium. It is also possible to

theorize that, given our differential effects on *S epidermidis* versus *S aureus*, for example, the specific organism may undergo species-specific membrane changes during incubation in Optisol, changes that might account for the differential effectiveness of the peptide.

An explanation of peptide activity, or lack thereof, on the surface of the eye is far more complex, and there may be multiple reasons for the failure of the peptide to work on the ocular surface despite its *in vitro* activity. One must take into account pharmacologic considerations such as dilutional factors, contact time, and retention time of the drug when delivered via topical application. In addition, the components of the tear film are very complex, including the lipid, aqueous, and mucin layers as well as glucose, lactate, citrate, glycoproteins, lysozyme, albumin, mucopolysaccharides, ions (Na, K, Mg, HCO<sub>3</sub>, HPO<sub>4</sub>), urea, amino acids, and sialic acid. Therefore, factors at the ocular surface, such as this complex mix of substances and the presence of proteases (both native and bacterial), could cleave or otherwise inactivate the synthetic antimicrobial peptide.

In addition, with the transition from an *in vitro* to an *in vivo* model, we must take into account a number of relevant issues, including our animal model (ie, bacterial load; incubation time prior to drug application; drug residence time, both ocular and systemic; *in vivo* immunologic factors; the isolates used) and issues of peptide and dosage delivery (pH, ionic strength, the presence of divalent ions, protease susceptibility, protein-protein interactions, membrane penetration, and pharmacokinetics).

Our *in vivo* model was developed after considerable experimentation with alternative approaches, and we designed it to yield a highly reproducible keratitis that mimicked the clinical circumstance (breach in the integrity of the epithelium plus topical challenge, rather than intrastromal injection). We intentionally chose a postinfection incubation time that we felt was comparable to the usual circumstance in human keratitis.

In our study, the rabbits uniformly progressed to corneal suppuration despite treatment with the peptide. We noted that the eyes sustained substantial damage by 96 hours, and this did not appear to be reversed in the animals tested with peptide. In the case of *P aeruginosa*, this rapid damage may be the result of potent proteases generated by the bacteria.<sup>233,234</sup> Bacteria-generated proteases may likewise be responsible for cleaving and rendering the peptide inactive. Alternatively, ocular surface proteins such as lysozyme might bind the peptide, making it inactive. In addition, *P aeruginosa* elaborates a mucoid biofilm that could conceivably serve as a barrier to the penetration of the peptide. In a previous study of the activity of defensins against *P aeruginosa*, Rich and colleagues<sup>235</sup> demonstrated that the *in vitro* antimicrobial

activity of rabbit defensin NP-3a was diminished in a linear fashion in the presence of human tears. A 3-log reduction was obtained in a test situation in which 10% tears were present. This was decreased to a 2-log reduction in the presence of 20% tears, and the peptide was rendered completely ineffective in the presence of 70% tears. These data are consistent with our findings of impaired activity at the ocular surface.

#### *Future Research Approaches to the Problem*

Each experiment, successful or unsuccessful, spawns new avenues of inquiry. Other considerations for investigating the role of peptides in the management of bacterial keratitis include alteration of the carrier media, including the use of a higher ionic strength carrier, more efficient buffering systems to maintain a pH that would favor potency of the peptide, and a more viscous carrier to increase drug residence time at the ocular surface. The hybridization of a peptide with a more traditional antibiotic such as polymixin is also a strategic option. In addition, we plan to (1) "dissect" the components of storage media and human tears to isolate inhibitory agents, (2) study peptide action over a longer period *in vitro*, and (3) further evaluate the kinetics of our animal model.

An alternative approach for the testing of *in vivo* effectiveness is a model using a "one-time" challenge in which the peptide and bacteria are mixed prior to the infection. While this does not mimic the clinical circumstance, such a model would help us distinguish between effectiveness of the peptide in the context of an infection and drug toxicity.

#### **THE FUTURE OF ANTIMICROBIAL PEPTIDES IN OPHTHALMOLOGY**

The past 50 years of the antibiotic era have witnessed an alarming increase in resistance to the natural, semisynthetic, and synthetic antibiotics.<sup>2,236</sup> The development of resistance as a phenomenon has been a reality since the widespread use of antibiotics, largely because of the short doubling times and genetic plasticity of microorganisms that allow them to test mutations that enhance their survivability.<sup>2</sup> The current "crisis" in antibiotic resistance is the result of widespread mutational changes that have brought about resistance to whole classes of antibiotics and the fact that no truly novel antibiotic agents have been developed in the past several decades.

Although we have relied on traditional antibiotic molecules like the penicillins, cephalosporins, aminoglycosides, and vancomycin for therapeutic purposes, it is interesting that most species throughout the evolutionary scale have employed peptide antimicrobials for immediate self-defense against pathogenic invasion. And yet, the employment of cationic peptide antimicrobials for the management of infectious diseases is at a very early stage

of development. Although peptide molecules clearly hold great promise for the management of infectious diseases in general and for ophthalmology specifically, indeed, very few *in vivo* studies of cationic peptide action have been published.<sup>237-239</sup> Certainly, companies involved in commercial development of these compounds have made claims of their *in vivo* applicability (eg, Applied Microbiology, Micrologix Biotech, Demeter Biotechnologies, Xoma, Magainin Pharmaceuticals, and Intrabiotics).

Theoretically, the use of the cationic peptides as antimicrobial agents has several distinct advantages:

1. They have the ability to effect killing of a broad spectrum of microorganisms, including multidrug-resistant bacteria, fungi, and even some enveloped viruses near the minimum inhibitory concentration (1 to 8  $\mu\text{g/mL}$ ), competitive with even the most potent antibiotics.
2. They kill much more rapidly than conventional antibiotics. (a 4- to 6-log reduction in survival often within 5 minutes,
3. They demonstrate a low level of resistance development *in vitro*. Since they operate on the basis of altering the permeability properties of the bacterial plasma membrane and do not interfere with cell wall or macromolecular synthesis as do traditional antibiotics, resistance to the peptide antimicrobials will not likely develop easily.
4. They have the ability in nature to protect animals against both topical and systemic infections and are able to neutralize endotoxins.<sup>237-240</sup>
5. They have demonstrated significant synergy with conventional antibiotics.<sup>238</sup>
6. Given the relatively small number of building blocks (approximately 20 amino acids on average), the peptides are quite amenable to synthesis and modification and, at the same time, offer tremendous diversity.<sup>2</sup>

However, there are some theoretical disadvantages to the use of peptides as clinical antimicrobials. Antimicrobial peptides have evolved in nature to be active within a specific and limited environment (eg, the neutrophil). Outside of this environment, the cationic peptides are programmed for deactivation so that they do not continue killing in other environments. Human defensins, outside the neutrophil, are deactivated by a variety of circumstances, such as change in ionic strength or binding to proteins. Therefore, the application of these substances in new environments (eg, the ocular surface) will require skirting those deactivating mechanisms placed so carefully by nature. In addition, peptide activity is not completely specific to prokaryotic cells, and the issues of toxicity to eukaryotic host cells need to be elaborated and resolved. Moreover, each natural peptide

has a broad but somewhat incomplete spectrum of activity, and in nature, these peptides often work together to mount an effective defense to bacterial invasion. Finding an effective application may require, therefore, the designing of a synthetic combination peptide that has a good minimal inhibitory concentration and that is nontoxic.

We face a present and emerging crisis in antibiotic therapy in which the extensive and, to some extent, indiscriminate use of antibiotics has precipitated significant resistance patterns and weakening of our armamentarium against bacterial infection.<sup>5</sup> Ophthalmic pharmaceutical companies have not rallied around the antimicrobial peptides yet, perhaps because of concern over production costs, *in vivo* stability, and unanticipated toxicity. However, the development of a new class of antimicrobials that have a broad spectrum of activity against serious ocular pathogens and a mechanism of action that would not likely generate patterns of resistance suggests that these peptides may be useful tools in the future for combating vision-threatening microbial infections of the eye. The application of the cationic peptides for the treatment of infectious disease in general and for the management of ocular infection specifically will require consideration of immunogenicity, toxicity, drug stability, formulation, and route of application. Successful achievement of this goal will require biochemical manipulation of these molecules to help overcome the problem of proteolysis that may reduce their half-life *in vivo*. Ophthalmologists and pharmacologists will need to collaborate in designing effective drugs that will achieve rapid eradication of pathogenic organisms without ocular cell toxicity. We are still at the beginning of the "peptide era." These compounds, elaborated by nature over millions of years as an effective defense system, are in their developmental infancy for application to eye disease. The future, nonetheless, holds promise.

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