The antimicrobial peptides produced by the so-called innate immune system are not only widely effective but may provide a new source of antibiotics

Innate Immunity: Ancient System Gets New Respect

In the deadly struggle between invading microbes and immune system defenses, antibodies and killer cells tend to get all the attention. But now appreciation is growing for another defense system that, until recently, has been largely ignored. Called innate immunity, the system is a kind of chemical barrier that organisms from insects to humans deploy to stop dangerous microbes at their first point of contact—the skin, mucous membranes, or other surfaces. This first line of defense has recently become a subject of intense study in several labs, because it represents a promising source of potential new antibiotic drugs. But the basic, primitive system is also turning out to be interesting in its own right.

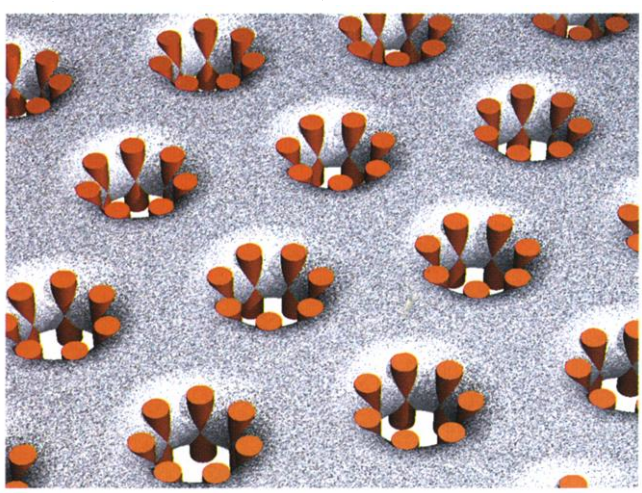
Unlike the so-called acquired immune system, with its disease-fighting cells and antibodies, innate immunity depends on peptides and small proteins to fight off dangerous microbes. As genomic information has flooded out of sequencing labs within the last few years, researchers have identified hundreds of these peptides from a broad range of species. They are also now working out exactly what trips these defenses and how they specialize to fight off the pathogens that attack each kind of organism.

As expected, the sugars and proteins that coat certain microbes are good triggers, but so too are various molecules that the infecting pathogens produce. Innate immunity "is quite a well-designed surveillance system whose extent, depth, complexity, and power have not been fully appreciated," says pediatrician and molecular biologist Michael Zasloff of Magainin Pharmaceuticals in Plymouth Meeting, Pennsylvania.

These defensive peptides tend to work very differently from conventional antibiotics, which generally block a crucial protein in an invading microbe. The peptides are less subtle killers: They punch holes in an invader's membranes or disrupt its internal signaling. Some even appear to pump up the host's own immune cell activity. As a result, most of the peptides are effective against a broad range of germs.

What's more, because the peptides home in on basic physical properties such as the overall charge on a microbial membrane, pathogens may be less likely to develop resistance. "It is hard for bacteria to change the physical properties of their membranes," notes Robert Hancock, a microbiologist at the University of British Columbia in Vancouver. "To develop resistance would entail that the microbes change their cells in very fundamental ways."

These attributes have caught the attention of the pharmaceutical industry. Clinical trials are already under way to see if the germ-killing peptides can combat everything from acne to catheter-related infections, the lung infections that plague cystic fibrosis patients, and the oral ulcers that often follow radiation and chemotherapy treatments for cancer. Early results look promising, although researchers caution that toxicity issues loom large, especially for molecules intended for internal use as opposed to topical application.



Pore former. Many antimicrobial peptides (shown here in orange) kill microbes by inserting in their membranes and opening pores.

CREDIT: L. YANG *ET AL., BIOPHYSICAL JOURNAL* 79, 2002 (2000)

Insects lead the way

Although research on antimicrobial peptides has intensified in the past 5 years, its roots can be traced back to the 1980s, when Hans Boman and his colleagues at the University of Stockholm, Sweden, sighted an intriguing phenomenon in pupae of the silkworm moth, *Hyalophora cecropia*. The researchers knew that insects, which do not have immune systems like those of higher animals, have some sort of innate immunity, but they were hazy about the details. To get at those, Boman's group inoculated the worms with bacteria and watched as they unleashed a batch of defensive agents. From that array, the team isolated two novel peptides, dubbed cecropin A and B, after the name of the moth.

The compounds, made from a string of 35 to 39 amino acids, appeared similar to a potent peptide called melittin found in bee venom. But Boman's curiosity was piqued when he noted that cecropins only kill bacteria such as *Escherichia coli*, while leaving moth cells unharmed. In contrast, melittin kills both bacteria and the cells of higher organisms. Boman thought he might be on to something interesting.

Over the next 2 decades, his group and others showed that antimicrobial peptides are extremely widespread in nature. They turned up in insects and eventually in higher species such as frogs and mammals, suggesting that innate immunity has deep evolutionary roots. Some, such as cecropin and attracin—another moth peptide identified by the Boman team—kill *E. coli* and certain other gram-negative bacteria (bacteria that fail to take up the so-called gram stain). Since then, researchers have identified other peptides that kill gram-positive bacteria. And still others, such as a peptide called drosocin, identified in the fruit fly by Jules Hoffmann's team at the Institute of Molecular and Cellular Biology at CNRS in Strasbourg, France, primarily attack fungi.

Hoffmann recalls that his group and others began studying fly defenses because even though the insects lack the cell-based immune systems seen in higher organisms, they are "extremely resistant to microorganisms." The large number of antimicrobial

peptides found in insects could go a long way toward explaining their resistance, as well as that of other organisms lacking advanced mammalian immune systems. Take frogs, for example.

In the late 1980s, Zasloff, then at the National Institutes of Health (NIH) in Bethesda, Maryland, found that frog skin harbors a potent defense system. When stimulated by injury or microbes, the animals sweat out large amounts of antibiotic peptides that Zasloff called "magainins" after the Hebrew word for "shield." In 1988, he left the NIH to launch a company, also named Magainin, to commercialize his discovery.

Peptide producer. When triggered by microbes or injury, frog skin secretes copious amounts of magainins.

But even animals with the most advanced immune systems produce defense peptides. In 1988, Boman's group purified cecropin-like peptides from the intestines of pigs. Robert Lehrer, along with Tomas Ganz and Michael Selsted of the University of California, Los Angeles, School of Medicine, discovered that other mammals, including rabbits, cows, and even humans, make similar antimicrobial peptides, which were named defensins. (Some insect and plant antimicrobial peptides are also called defensins, although their structures are different from those of the mammalian peptides.) Most recently, a team led by Yong-Lian Zhang at the Shanghai Institute for Biological Sciences in China identified a new defensin in the reproductive tract of male rats that may aid sperm development and help guard against infection (*Science*, 2 March, p. 1783).

In fact, in higher animals, cells in and around the linings of the intestines and of the respiratory and urogenital tracts commonly churn out the peptides. So do phagocytic cells called neutrophils, which use defensins to kill the microbes they engulf. The potent molecules are produced at low levels all the time. But Zasloff and others, such as Ganz, have found that injury or inflammatory signals such as interleukin-1β can cause defensin production to skyrocket in the alimentary tract of cows. "We have the basis of a shield that is not just sitting there passively but is capable of induction," Zasloff says.

The peptides are proving to be vital for survival. For instance, in work reported on page 113 of the 1 October 1999 issue of *Science*, Carole Wilson of the Washington University School of Medicine in St. Louis and Andre Ouellette of the University of California, Irvine, genetically engineered mice to be defective in an enzyme needed to activate a defensin produced by cells located at the base of the tiny finger-like projections lining the small intestine. Researchers had known for decades that the cells maintain a "sterile" microenvironment, but they didn't know how. The knockout mice gave some clues. They seemed normal at birth, but when the animals were infected with disease-causing strains of either *E. coli* or *Salmonella* bacteria, they succumbed more readily to illness, even death. The same susceptibility to infection occurs in flies that have had their peptide defenses knocked out.

How the peptides work

Even as the number of antimicrobial peptides has continued to swell, researchers have been trying to figure out how they work. Boman and Stockholm colleague Dan Hultmark showed early on that some, such as the cecropins and insect or mammalian defensins, seem to home in on and rupture the negatively charged membranes of bacteria. But because the peptides pick out some bacteria and not others with a similar charge, researchers are sure there is more to the story. They also note that the peptides don't damage the cells of the organisms that make them. "It's amazing," says Zasloff. "Despite being so broad spectrum and working on membranes, these antimicrobial peptides don't harm the host."

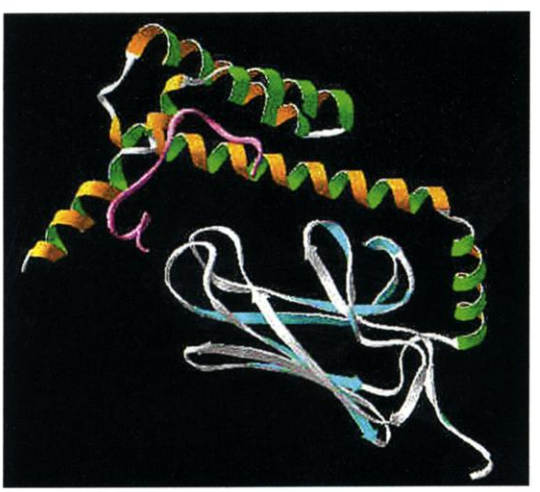
So far, the explanation for this remains a mystery. Some investigators have suggested that host cells may carry a lower negative charge than the microbes. But charge differences alone can't explain how some peptides can distinguish among members of the same class of germs. Another possibility is that rather than just recognizing net charge, the peptides home in on patterns of charge, say in the sugars or amino acid chains that sit on a microbe's surface.

In one special case, researchers do have a clear idea of how a peptide works. In 1994, Hoffmann and his colleagues identified an antimicrobial peptide in the European sap-sucking bug, *Pyrrhocoris apterus*, that acts very differently from previously discovered antimicrobial peptides. Those tend to kill quickly and to act either on gram-positive or gram-negative bacteria. But pyrrhocoricin takes anywhere from 6 to 12 hours to work and kills representatives from both classes of bacteria. That suggests that the peptide targets a protein common to different classes of bacteria. In October, Laszlo Otvos and his colleagues at the Wistar Institute in Philadelphia fingered that target: a protein called DnaK that normally works to preserve the three-dimensional shapes of many proteins in the face of heat. Interfering with DnaK destroys many enzymes involved in normal microbial housekeeping, thus killing the organisms.

Researchers are also getting a handle on just what triggers the production of the peptides. While investigating how fungi spur the production of peptides such as drosocin, Hoffmann and others found that elusive molecular signals produced by the microbes trigger the so-called "Toll" pathway to turn on the genes needed to synthesize the peptides (*Science*, 25 September 1998, p. 1942). The pathway is named after the Toll protein, a receptor that sits in the cell membrane, where it can pick up signals from the outside. Until then, Toll was known only for its role in regulating early fly embryo development.

Researchers have since learned that the fly carries at least eight Toll receptor variants, each of which seems to be triggered by a specific class of attacking microbe. One Toll family receptor can pick out, say, fungi, while another detects gram-positive bacteria. The activated receptor then signals the cell nucleus to make the appropriate peptide to combat that type of microbe.

Researchers, including Charles Janeway of Yale University in New Haven, Connecticut, and Shizuo Akira and colleagues at Osaka University in Japan, have shown that something similar happens in human cells. For example, Janeway demonstrated that endotoxin, a lipid- and sugar-laden toxin made by gram-



All tied up. Unlike most antimicrobial peptides, which punch holes in membranes, pyrrhocoricin (purple) kills its targets by binding to an internal protein called DnaK.

CREDITS: (TOP TO BOTTOM) MAGAININ PHARMACEUTICALS; G. KNAGOL *ET AL.*, *BIOCHEMISTRY* 40, 3016 (2001)

Engineering Protection for Plants

Animals aren't the only creatures that need potent defenses against microbial pathogens. Plants need them as well. Now, researchers are trying to use antimicrobial peptides originally found in insects and other animals (see main text) to boost plants' ability to resist infectious bacteria and fungi. Current targets include the potato blight fungus, which caused the great Irish famine of the mid-1800s, and the bacterium that causes soft rot in stored potatoes.

In previous efforts, researchers tried to bolster plant defenses by rubbing antimicrobial peptides from insects on their leaves or by genetically engineering plants to make the peptides internally. But those approaches failed, because the insect peptides proved toxic to the plants. To get around this problem, plant geneticist Santosh Misra and microbiologist William Kay of the University of Victoria in British Columbia tried making less toxic peptides, using a "mix and match" strategy devised with the help of microbiologist Robert Hancock of the University of British Columbia in Vancouver.

In work reported in the November issue of *Nature Biotechnology*, Misra constructed hybrid DNAs that combined DNA sequences from moth and bee peptide genes. Test tube studies showed that some of the resulting peptides retained the ability to kill bacteria without harming potato cells. Misra's team then shuttled the DNA encoding the best of these into Russet Burbank and Desiree potatoes with the help of *Agrobacterium tumefaciens*, a bacterium that injects genetic material into plant cells.

Resistant. Fungal infection causes the control potato plant (left) to shrivel and die, while the defensin-making plant (right) continues to grow.

Once the genetically altered potato plants reached 2 weeks of age, the British Columbia team exposed them to the blight-causing fungi. The microbes infected unaltered potato plants within 11 days, but the modified Desiree plants grew just fine, even through a cloud of fungi clustered about their roots. The genetically altered Russet Burbank plants didn't do quite as well, however. Although they proved more resistant to infection than controls, they grew yellow, curly leaves and smaller, branched tubers than did normal Russets. The researchers don't know what accounts for this difference.

In a second set of studies, the researchers exposed sliced potato tubers from both genetically modified and control plants to *Erwinia carotova*, the bacterium that causes soft rot. Exposed controls were quickly infected and lost 60% of their weight. In contrast, tubers from the altered potato plants appeared similar to uninfected controls. They could also withstand storage for at least 6 months, while controls succumbed to soft rot. What's more, when fed to mice, the peptide-producing potatoes were as benign to the rodents as control tubers.

In similar studies, Ai-Guo Gao's team at Monsanto in St. Louis reported in the December issue of *Nature Biotechnology* that Russet potatoes expressing modified alfalfa antifungal peptides showed enhanced resistance to the fungus that causes "early dying" disease. The Monsanto team also found that the modified plants retained their resistance to the pathogen in field trials.

The British Columbia team is now testing its defense-enhanced potatoes in field trials, and Misra estimates that the tubers could make it to supermarket shelves in 3 to 5 years—provided they aren't stalled by public reluctance to accept genetically modified foods. Indeed, such concerns have caused plant pathologist Herbert Aldwinckle at the Geneva campus of Cornell University to decide against commercializing a genetically modified apple strain his group produced. The trees carry a variant of the antimicrobial peptide attacin, which has worked well at protecting them from the bacterial disease fire blight. "I think these antimicrobials do have the potential to work in crops in the field," Aldwinckle says. But even if the crops are safe, he says, "we are very concerned [that] anything we put into apple might reflect badly on the image of apple as a crop." **–T.G.**

negative bacteria such as *E. coli* and *Salmonella typhimurium*, prompts Toll-like receptors in human cells to trigger the production of mammalian defensins. To date, at least nine human Toll-like receptors (TLRs) have been identified, each of which may recognize a distinct pattern of molecules characteristic of a class of microbes. For example, TLR4 seems to trigger a defense cascade in response to endotoxin, while TLR2 recognizes components from the walls of gram-positive bacteria such as *Listeria monocytogenes*.

Into the clinic

Whatever drives the production of so many different antimicrobial peptides, they are providing a bonanza of potential agents for treating human infections. Still, the researchers caution against overoptimism: If antimicrobial peptides are not specific enough, they can overzealously target human cell membranes or rupture red blood cells, leading to rapid toxicity, even death, for the host, as has been found in animals.

Investigators are now exploring several ways to circumvent these toxicity problems —and they are seeing some signs of success. One possibility is to screen for natural peptides that spare host tissues. The fruit fly peptide drosocin, for example, may have the type of specificity desired. "If you take peptides, which have been selected by nature for hundreds of millions of years, you're bound to find some that are not cytotoxic," Hoffmann says.

To develop that idea—and possible candidates—the molecular biologist has spun off a company in Strasbourg called EntoMed. Headed by Hoffmann's former Strasbourg colleague Jean-Luc Demarcq, the company is now conducting studies in rodents to see whether drosocin can combat two fungi, *Candida albicans* and *Aspergillus fumigatus*, that cause often fatal infections in patients undergoing chemotherapy or organ transplants. Demarcq says the company hopes to move into human studies by 2002.

Meanwhile, Intrabiotics in the San Francisco Bay area of California is conducting clinical trials to see whether a peptide spawned by pig neutrophils can treat mouth ulcers in patients undergoing chemotherapy or radiotherapy for head and neck cancers. Those trials are in their final stages, and Intrabiotics has also begun testing whether the peptide in aerosol form can prevent the *Pseudomonas* infections that often kill cystic fibrosis patients and the pneumonia that afflicts patients on ventilators.

Other researchers, including Hancock in Vancouver and Lehrer in Los Angeles, are trying to produce safer peptides by modifying some of the more potent ones to dampen their activity. They either synthesize the variants from scratch or use recombinant DNA technology to produce modified peptides in bacteria. The researchers add the peptide candidates to test tubes filled with various strains of bacteria and sheep red blood cells, and look for those that kill the bacteria without damaging the blood cells.

CREDIT: M. OSUSKY *ET AL.*, *NATURE BIOTECHNOLOGY* 18, 1162 (2000)

Already, this strategy has produced three peptides that have moved into clinical trials. None are being injected systemically, however, because the researchers consider that route of administration too risky right now. Micrologix Biotech Inc. in Vancouver is currently conducting a late-stage clinical trial of one of these peptides, aimed at seeing whether it will prevent the catheter-related infections caused by many microbes, including the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*, a gram-positive superbug that now resists most common current antibiotics. Company researchers are also testing whether the peptide will cure severe acne and prevent acute *S. aureus* infections.

Zasloff's team at Magainin is taking another tack: trying to get the body to produce more of its own defensive peptides, which presumably won't damage the cells of their native host. The team began by looking for potential triggers in disease-causing bacteria such as *Staphylococcus* and *Pseudomonas*. Those pathogens did not boost β-defensin gene expression. But much to Zasloff's surprise, beneficial organisms, such as bakers' yeast and *Lactobacillus*, found in yogurt, did. The team then purified the active component from bakers' yeast, which turned out to be the amino acid isoleucine. The amino acid may work by binding to a Toll-like receptor in people, the researchers reported in the *Proceedings of the National Academy of Sciences* in November.

Although isoleucine is a necessary building block of the body's proteins, humans cannot make the amino acid on their own and must get it in their diets. Zasloff suggests that isoleucine is a good signal of an invading pathogen, because the microbes do make the amino acid and would drive isoleucine levels up during infection—either by secreting it or by breaking down host tissue. If so, then something as simple as an amino acid supplement might boost immunity. But it remains to be seen whether such a strategy will be successful in designing actual therapies.

Even if the peptides now being tested don't pan out, there are plenty of other candidates waiting in the wings. "It is becoming clear that the hard-wiring of an animal, even the mammal, can be fine-tuned to very carefully distinguish between different classes of microbe," Zasloff says. **–TRISHA GURA**

Trisha Gura is a science writer in Cleveland, Ohio.

MEETING BIOPHYSICAL SOCIETY

Crossover Research Yields Scents and Sensitivity

BOSTON, MASSACHUSETTS—Over 5000 scientists gathered here last month for the 45th annual meeting of the Biophysical Society.* The meeting brought together physicists, chemists, biologists, and others to discuss how physics can be used to address fundamental biological problems in new ways.

Watching a Virus Get Stuffed

For decades, scientists have used viruses that infect bacteria, known as phages, as models for viruses that infect humans. Yet despite intense scrutiny, how phages manufacture more phages remains mysterious. Now, for the first time, biophysicists from the University of California (UC), Berkeley, have caught a phage in a key act of self-assembly. Using microscopic beads and laser light, they watched the virus stuffing its genome into a protein shell, and they played tug-of-war with its DNA. The results reveal that the microscopic winch responsible for DNA packaging is the most powerful molecular motor ever measured.

"They've done a remarkable job," says Dwight Anderson, a phage biologist at the University of Minnesota, Minneapolis. "For a long time, we've been using an integrated biochemical and genetic approach [to understand packaging], but what we really needed is the biophysics."

The DNA packaging machinery of the phage, known as phi29, has been studied extensively. To stuff DNA into the virus's five-sided head, a phage protein called gp16 brings the DNA to a protein-RNA complex at the base of the head. The gp16 burns up adenosine triphosphate (ATP) fuel and, together with the protein connector, pulls the DNA into the head. Enlarged 600,000 times, the whole process would resemble stuffing a strand of spaghetti into a matchbox. But although its biochemistry is well known, the mechanical details of how the virus pulls the DNA into the head and organizes it in just a few minutes are sketchy.

To get a handle on the problem, Sander Tans of UC Berkeley literally took hold of the phage while it packaged. He and colleagues Doug Smith, Steve Smith, and Carlos Bustamante strung a phage head and DNA between two small plastic beads, attaching them with a protein glue. Using suction, the researchers held the bead with the phage head on the end of a micropipette. The DNA bead, meanwhile, was caught in a laser trap—a specially focused laser beam that holds the bead stationary. By pulling on the DNA–phage head assembly and measuring the deflection of the laser trap's light, the researchers could measure the force on the bead—and thus on the biological molecules attached to it. After assembling the complex, they added ATP and watched.

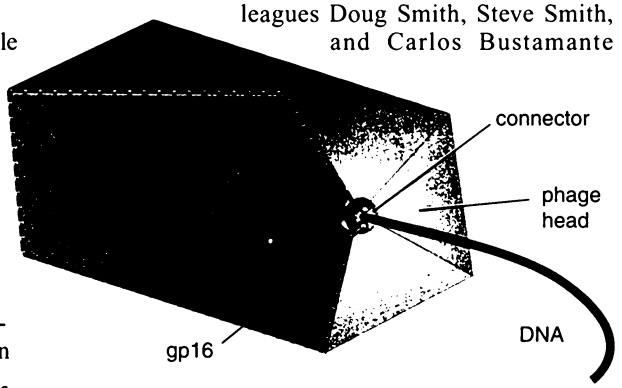


ILLUSTRATION: J. MOGLIA

Packaging power. The protein motor that stuffs DNA into virus heads is the strongest yet.

As the motor pulled the DNA into the phage head, the researchers saw the beads moving closer and closer together. The DNA packaging began smoothly, at a constant rate of 100 base pairs per second. But once the head was half full of the DNA, the pressure began to build up and the motor to slow down. Tans and his co-workers used the optical trap to pull against the packaging motor. They could bring the motor to a stop by resisting with a force of 57 piconewtons—the highest "stalling force" ever seen for a molecular motor. From that force and the volume inside a phage head, Tans calculates that the pressure inside a fully stuffed head is 15 megapascals. "That's the pressure inside an oxygen bottle," he says. He conjectures that the high pressure may help the phage inject its DNA when it attaches to the outside of a cell it's going to infect.

A thorough understanding of packaging, Anderson says, could reveal a new target for novel antiviral drugs. Other scientists are also excited by the application of biophysical techniques to the problem. Without them, "we would never learn what a strong engine the virus packaging machinery has," says Roman Tuma of the University of Helsinki in Finland.

* 17 to 21 February 2001, Hynes Convention Center, Boston, Massachusetts.