

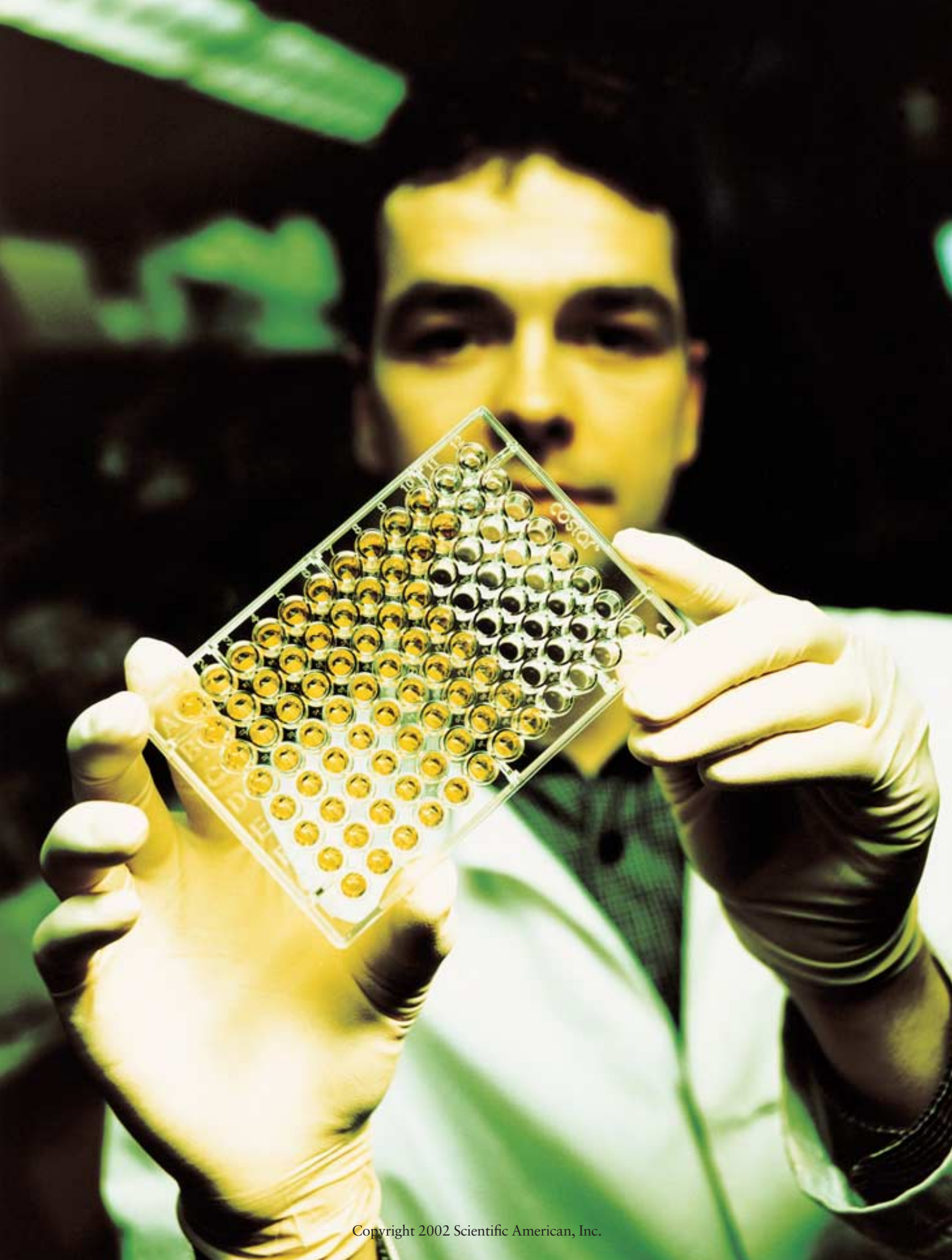
ATTACKING ANTHRAX

Recent discoveries are suggesting much-needed strategies for improving prevention and treatment.

High on the list: ways to neutralize the anthrax bacterium's fiendish toxin

by John A. T. Young and R. John Collier

CULTURES OF CELLS survived exposure to the anthrax toxin after being treated with a potential antitoxin. Michael Mourez of Harvard University holds a plate containing the cultures.



The need for new anthrax therapies became all too clear last fall

when five people died of inhalation anthrax, victims of the first purposeful release of anthrax spores in the U.S. Within days of showing initially unalarming symptoms, the patients were gone, despite intensive treatment with antibiotics. Six others became seriously ill as well before pulling through.

Fortunately, our laboratories and others began studying the causative bacterium, *Bacillus anthracis*, and seeking antidotes long before fall 2001. Recent findings are now pointing the way to novel medicines and improved vaccines. Indeed, in the past year alone, the two of us and our collaborators have reported on three promising drug prototypes.

An Elusive Killer

THE NEW IDEAS for fighting anthrax have emerged from ongoing research into how *B. anthracis* causes disease and death. Anthrax does not spread from individual to individual. A person (or animal) gets sick only after incredibly hardy spores enter the body through a cut in the skin, through contaminated food or through

spore-laden air. Inside the body the spores molt into “vegetative,” or actively dividing, cells.

Anthrax bacteria that colonize the skin or digestive tract initially do damage locally and may cause self-limited ailments: black sores and swelling in the first instance; possibly vomiting and abdominal pain and bleeding in the second. If bacterial growth persists unchecked in the skin or gastrointestinal tract, however, the microbes may eventually invade the bloodstream and thereby cause systemic disease.

Inhaled spores that reach deep into the lungs tend to waste little time where they land. They typically convert to the vegetative form and travel quickly to lymph nodes in the middle of the chest, where many of the cells find ready access to the blood. (Meanwhile bacteria that remain in the chest set the stage for a breath-robbing buildup of fluid around the lungs.)

Extensive replication in the blood is generally what kills patients who succumb to anthrax. *B. anthracis*'s ability to expand so successfully derives from its

secretion of two substances, known as virulence factors, that can profoundly derail the immune defenses meant to keep bacterial growth in check. One of these factors encases the vegetative cells in a polymer capsule that inhibits ingestion by the immune system's macrophages and neutrophils—the scavenger cells that normally degrade disease-causing bacteria. The capsule's partner in crime is an extraordinary toxin that works its way into those scavenger cells, or phagocytes, and interferes with their usual bacteria-killing actions.

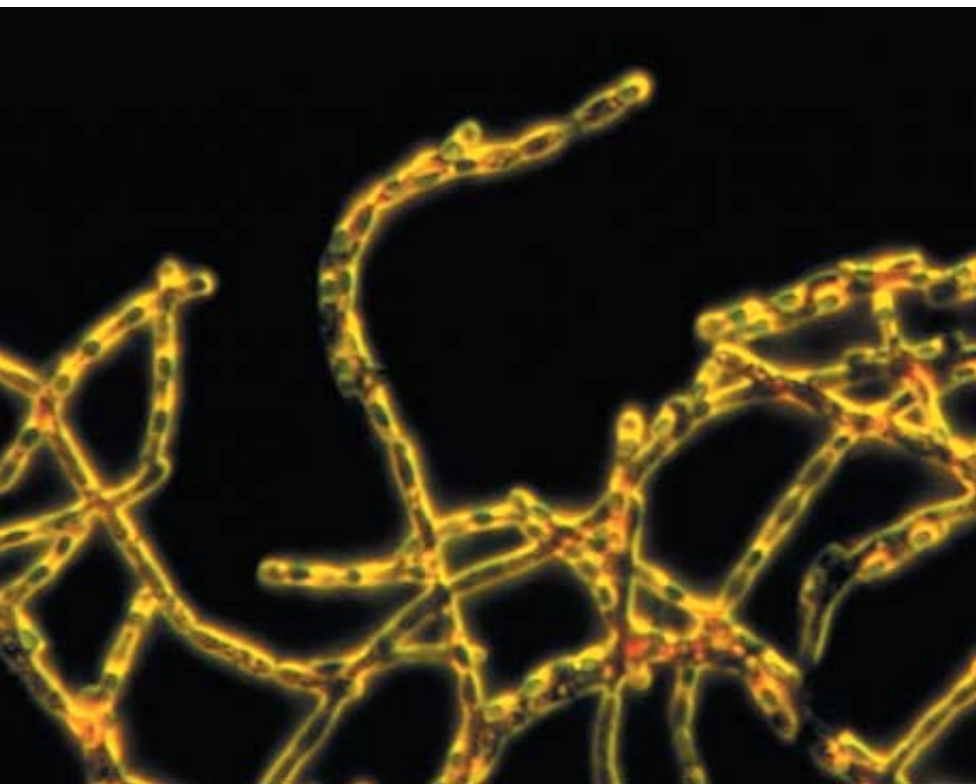
The anthrax toxin, which also enters other cells, is thought to contribute to mortal illness not only by dampening immune responses but also by playing a direct role. Evidence for this view includes the observation that the toxin alone, in the absence of bacteria, can kill animals. Conversely, inducing the immune system to neutralize the toxin prevents *B. anthracis* from causing disease.

A Terrible Toxin

HARRY SMITH and his co-workers at the Microbiological Research Establishment in Wiltshire, England, discovered the toxin in the 1950s. Aware of its central part in anthrax's lethality, many researchers have since focused on learning how the substance “intoxicates” cells—gets into them and disrupts their activities. Such details offer essential clues to blocking its effects. Stephen H. Leppla and Arthur M. Friedlander, while at the U.S. Army Medical Research Institute of Infectious Diseases, initiated that effort with their colleagues in the 1980s; the two

Overview/*Anthrax*

- A three-part toxin produced by the anthrax bacterium, *Bacillus anthracis*, contributes profoundly to the symptoms and lethality of anthrax.
- The toxin causes trouble only when it gets into the cytosol of cells, the material that bathes the cell's internal compartments.
- Drugs that prevented the toxin from reaching the cytosol would probably go a long way toward limiting illness and saving the lives of people infected by the anthrax bacterium.
- Analyses of how the toxin enters cells have recently led to the discovery of several potential antitoxins.



ACTIVELY DIVIDING CELLS of the anthrax bacterium arrange themselves into chains that resemble linked boxcars.

of us and others took up the task somewhat later.

The toxin turns out to consist of three proteins: protective antigen, edema factor and lethal factor. These proteins cooperate but are not always joined together physically. They are harmless individually until they attach to and enter cells, which they accomplish in a highly orchestrated fashion.

First, protective antigen binds to the surface of a cell, where an enzyme trims off its outermost tip. Next, seven of those trimmed molecules combine to form a ring-shaped structure, or heptamer, that

captures the two factors and is transported to an internal membrane-bound compartment called an endosome. Mild acidity in this compartment causes the heptamer to change shape in a way that leads to the transport of edema factor and lethal factor across the endosomal membrane into the cytosol (the internal matrix of cells), where they do their mischief. In essence, the heptamer is like a syringe loaded with edema factor and lethal factor, and the slight acidity of the endosome causes the syringe to pierce the membrane of the endosome and inject the toxic factors into the cytosol.

Edema factor and lethal factor catalyze different molecular reactions in cells. Edema factor upsets the controls on ion and water flow across cell membranes and thereby promotes the swelling of tissues. In phagocytes, it also saps energy that would otherwise be used to engulf bacteria.

The precise behavior of lethal factor, which could be more important in causing patient deaths, is less clear. Scientists do know that it is a protease (a protein-cutting enzyme) and that it cleaves enzymes in a family known as MAPKKs. Now they are trying to tease out the molecular events that follow such cleavage

and to uncover the factor's specific contributions to disease and death.

Therapeutic Tactics

CERTAINLY DRUGS able to neutralize the anthrax toxin would help the immune system fight bacterial multiplication and would probably reduce a patient's risk of dying. At the moment, antibiotics given to victims of inhalation anthrax may control microbial expansion but leave the toxin free to wreak havoc.

In principle, toxin activity could be halted by interfering with any of the steps in the intoxication process. An attractive approach would stop the sequence almost before it starts, by preventing protective antigen from attaching to cells. Scientists realized almost 10 years ago that this protein initiated toxin entry by binding to some specific protein on the surface of cells; when cells were treated with enzymes that removed all their surface proteins, protective antigen found no footing. Until very recently, though, no one knew which of the countless proteins on cells served as the crucial receptor.

The two of us, with our colleagues Kenneth Bradley, Jeremy Mogridge and Michael Mourez, found the receptor last summer. Detailed analysis of this molecule (now named ATR, for anthrax toxin receptor) then revealed that it spans the cell membrane and protrudes from it. The protruding part contains an area resembling a region that serves in other receptors as an attachment site for particular proteins. This discovery suggested that the area was the place where protective antigen latched onto ATR, and indeed it is.

We have not yet learned the normal function of the receptor, which surely did not evolve specifically to allow the anthrax toxin into cells. Nevertheless, knowledge of the molecule's makeup is enabling us to begin testing inhibitors of its activity. We have had success, for instance, with a compound called sATR, which is a soluble form of the receptor domain that binds to protective antigen. When sATR molecules are mixed into the medium surrounding cells, they serve as effective decoys, tricking protective antigen into binding to them instead of to its true receptor on cells.

THE AUTHORS

JOHN A. T. YOUNG and **R. JOHN COLLIER** have collaborated for several years on investigating the anthrax toxin. Young is Howard M. Temin Professor of Cancer Research in the McArdle Laboratory for Cancer Research at the University of Wisconsin–Madison. Collier, who has studied anthrax for more than 14 years, is Maude and Lillian Presley Professor of Microbiology and Molecular Genetics at Harvard Medical School.

Detecting Anthrax

Rapid sensing would save lives

By Rocco Casagrande

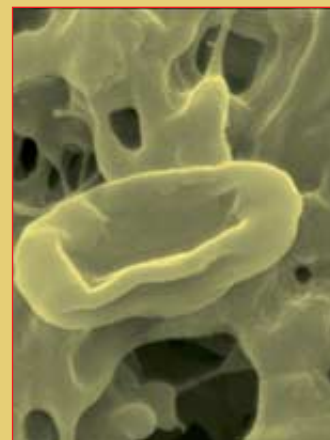
IF A TERRORIST GROUP spread anthrax spores into the open air, the release could affect large numbers of people but would probably go unnoticed until victims showed up at hospitals. Many would undoubtedly seek help too late to be saved by current therapies. Much illness could be prevented, however, if future defenses against anthrax attacks included sensors that raised an alarm soon after spores appeared in the environment. The needed instruments are not yet ready for deployment, but various designs that incorporate cutting-edge technology are being developed.

Environmental sensors must discriminate between disease-causing agents (pathogens) and the thousands of similar but harmless microorganisms that colonize air, water and soil. Most of the tools being investigated work by detecting unique molecules on the surface of the pathogens of interest or by picking out stretches of DNA found only in those organisms.

The Canary, which is being developed at the Massachusetts Institute of Technology Lincoln Laboratory, is an innovative example of the devices that detect pathogens based on unique surface molecules. The sensors of the Canary consist of living cells—B cells of the immune system—that have been genetically altered to emit light when their calcium levels change. Protruding from these cells are receptors that will bind only to a unique part of a surface molecule on a particular pathogen. When the cells in the sensor bind to their target, that binding triggers the release of calcium ions from stores within the cells, which in turn causes the cells to give off light. The Canary can discern more than one type of pathogen by running a sample through several cell-filled modules, each of which reacts to a selected microorganism.

The GeneXpert system, developed by Cepheid, in Sunnyvale, Calif., is an example of a gene-centered approach. It begins its work by extracting DNA from microorganisms in a sample. Then, if a pathogen of concern is present, small primers (strips of genetic material able to recognize specific short sequences of DNA) latch onto the ends of DNA fragments unique to the pathogen. Next, through a procedure called the polymerase chain reaction (PCR), the system makes many copies of the bound DNA, adding fluorescent labels to the new copies along the way. Within about 30 minutes GeneXpert can make enough DNA to reveal whether even a small amount of the worrisome organism inhabited the original sample.

This system contains multiple PCR reaction chambers with



CARTRIDGE used in the experimental GeneXpert system is about as tall as an adult's thumb (*left*). Inside, sound waves bombard material to be tested, causing any cells to break open and release their DNA. If a pathogen of interest is present, its DNA will be amplified in the arrow-shaped reaction tube (*protrusion*), and the edges of the arrowhead will fluoresce. The micrograph (*right*) shows the remains of a cell that has disgorged its contents.

distinct primer sets to allow the detection of different pathogens simultaneously. Furthermore, the GeneXpert system could be used to determine whether the anthrax bacterium is present in a nasal swab taken from a patient in as little as half an hour, significantly faster than the time it takes for conventional microbiological techniques to yield results.

Instruments designed specifically to detect spores of the anthrax bacterium or of closely related microbes (such as the one that causes botulism) can exploit the fact that such spores are packed full of dipicolinic acid (DPA)—a compound, rarely found elsewhere in nature, that helps them to survive harsh environmental conditions. Molecules that fluoresce when bound to DPA have shown promise in chemically based anthrax detectors. “Electronic noses,” such as the Cyranose detection system made by Cyranose Sciences in Pasadena, Calif., could possibly “smell” the presence of DPA in an air sample laced with anthrax spores.

The true danger of an anthrax release lies in its secrecy. If an attack is discovered soon after it occurs and if exposed individuals receive treatment promptly, victims have an excellent chance of surviving. By enhancing early detection, sensors based on the systems discussed above or on entirely different technologies could effectively remove a horrible weapon from a terrorist's arsenal.

ROCCO CASAGRANDE is a scientist at Surface Logix in Brighton, Mass., where he is developing methods and devices for detecting biological weapons.

We are now trying to produce sATR in the amounts needed for evaluating its ability to combat anthrax in rodents and nonhuman primates—experiments that must be done before any new drug can be considered for fighting anthrax in people. Other groups are examining whether carefully engineered antibodies (highly specific molecules of the immune system)

might bind tightly to protective antigen in ways that will keep it from coupling with its receptor.

More Targets

SCIENTISTS ARE ALSO seeking ways to forestall later steps in the intoxication pathway. For example, a team from Harvard has constructed a drug able to clog

the regions of the heptamer that grasp edema and lethal factors. The group—from the laboratories of one of us (Collier) and George M. Whitesides—reasoned that a plugged heptamer would be unable to draw the factors into cells.

We began by screening randomly constructed peptides (short chains of amino acids) to see if any of them bound to the

ANTHRAX IN ACTION

Physicians classify anthrax according to the tissues that are initially infected. The disease turns deadly when the causative bacterium, *Bacillus anthracis*, reaches the bloodstream and proliferates there, producing large amounts of a dangerous toxin. Much research is now focused on neutralizing the toxin.

THREE TYPES

INHALATION ANTHRAX
Spores are breathed in

GASTROINTESTINAL ANTHRAX
Spores are ingested by eating contaminated meat

CUTANEOUS ANTHRAX
Spores penetrate the skin through a break

HOW INHALATION ANTHRAX ARISES

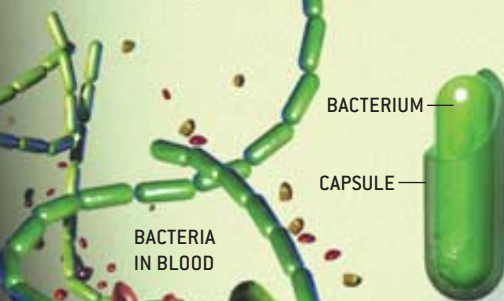
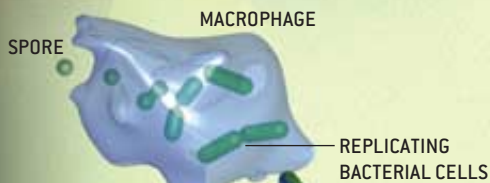
Inhalation anthrax is the most dangerous form, probably because bacteria that land in the lungs are more likely to reach the bloodstream and thus disseminate their toxin through the body.

1 Immune system cells called macrophages ingest *B. anthracis* spores and carry them to lymph nodes in the chest. En route, or in the macrophages, the spores transform into actively dividing cells

2 Proliferating *B. anthracis* cells erupt from macrophages and infiltrate the blood readily

3 In the blood, the active bacteria evade destruction by macrophages and other cells of the immune system by producing a capsule [detail] that blocks the immune cells from ingesting them and by producing a toxin that enters immune cells and impairs their functioning

4 Protected from immune destruction, the bacteria multiply freely and spread through the body



MACROPHAGE FILLED WITH TOXIN

TOXIN MOLECULES

HOW THE TOXIN INVADES CELLS ... AND HOW TO STOP IT

THE ANTHRAX TOXIN must enter cells to hurt the body. It consists of three collaborating proteins: protective antigen (PA), edema factor (EF) and lethal factor (LF). The last two disrupt cellular activities, but only after protective antigen delivers them to the cytosol—the matrix surrounding the cell's intracellular compartments. Molecular understanding of how the factors reach the cytosol has led to ideas for blocking that journey and thus for neutralizing the toxin and saving lives. The antitoxins depicted in the boxes have shown promise in laboratory studies.



1 PA binds to its receptor on a cell

2 PA gets cleaved

3 Seven copies combine, forming a heptamer

4 Up to three copies of EF or LF or a combination of the two bind to the heptamer

TREATMENT IDEA

INHIBITOR

Keep EF and LF from attaching to their binding sites on PA heptamers. Plug those sites with linked copies of a molecule that also has affinity for the sites.

5 The heptamer complexed with EF and LF is delivered to a membrane-bound compartment called an endosome

6 Mild acidity in the endosome causes the heptamer to inject EF and LF into the cytosol

TREATMENT IDEA

ENDOSOME

HEPTAMER

DNI

CYTOSOL

Block transport of EF and LF from the endosome into the cytosol by causing newly forming heptamers to incorporate a version of PA known as a dominant negative inhibitor (DNI). DNI-containing heptamers cannot move EF and LF across the endosome's membrane.

TREATMENT IDEA

SOLUBLE RECEPTOR (sATR)

PA

Prevent PA from linking to its receptor on cells. Induce it to bind instead to decoys, such as soluble copies of the toxin receptor's PA binding site.

8 LF is believed to be important in causing disease and death, but exactly how it does so is in question

7 EF causes tissues to swell and prevents immune system cells from ingesting and degrading bacteria

CYTOSOL

heptamer. One did, so we examined its ability to block toxin activity. It worked, but weakly. Assuming that fitting many plugs into the heptamer's binding domains for edema and lethal factor would be more effective, we took advantage of chemical procedures devised by Whitesides's group and linked an average of 22 copies of the peptide to a flexible polymer. That construction showed itself to be a strong inhibitor of toxin action—more than 7,000 times better than the free peptide—both in cell cultures and in rats.

And understanding of the receptor's three-dimensional structure would reveal the precise contact points between protective antigen and the receptor, enabling drugmakers to custom-design receptor blocking agents.

Scientists would also like to uncover the molecular interactions that enable protective antigen heptamers to move from the cell surface into endosomes inside the cell. Impeding that migration should be very useful. And what happens after lethal factor cleaves MAPKK en-

tralize the toxin of concern as soon as it appears in the body, thus preventing disease. Livestock in parts of the U.S. receive preparations consisting of *B. anthracis* cells that lack the protective capsule and thus replicate poorly. A similar vaccine for humans has been used in the former Soviet Union. But preparations that contain whole microbes often cause side effects, and they raise the specter that renegade cells might at times give rise to the very diseases they were meant to prevent.

The only anthrax vaccine approved

To be most effective, antitoxins will probably be USED WITH ANTIBIOTICS, much as cocktails of antiviral drugs are recommended for treating HIV infection.

Another exciting agent, and the one probably closest to human testing, would alter the heptamer itself. This compound was discovered after Bret R. Sellman in Collier's group noted that when certain mutant forms of protective antigen were mixed with normal forms, the heptamers formed on cells as usual but were unable to inject edema and lethal factors into the cytosol. Remarkably, some of these mutants were so disruptive that a single copy in a heptamer completely prevented injection.

In a study reported last April, these mutants—known as dominant negative inhibitors, or DNIs—proved to be potent blockers of the anthrax toxin in cell cultures and in rats. Relatively small amounts of selected DNIs neutralized an amount of protective antigen and lethal factor that would otherwise kill a rat in 90 minutes. These findings suggest that each mutant copy of protective antigen is capable of inactivating six normal copies in the bloodstream and that it would probably reduce toxin activity in patients dramatically.

Of course, as more and more questions about the toxin are answered, scientists should discover further treatment ideas. Now that the receptor for protective antigen has been identified, researchers can use it as a target in screening tests aimed at finding drugs able to bar the receptor from binding to protective antigen.

zymes? How do those subsequent events affect cells? Although the latter question remains a vexing challenge, recent study of lethal factor has brightened the prospects for finding drugs able to inactivate it. Last November, Robert C. Liddington of the Burnham Institute in La Jolla, Calif., and his colleagues in several laboratories published the three-dimensional structure of the part of lethal factor that acts on MAPKK molecules. That site can now become a target for drug screening or design.

New leads for drugs should also emerge from the recent sequencing of the code letters composing the *B. anthracis* genome. By finding genes that resemble those of known functions in other organisms, biologists are likely to discover additional information about how the anthrax bacterium causes disease and how to stop it.

The continuing research should yield several antitoxins. To be most effective, such drugs will probably be used with antibiotics, much as cocktails of antiviral drugs are recommended for treating HIV infection.

Promising Preventives

AS PLANS TO IMPROVE therapies proceed, so does work on better vaccines. Vaccines against toxin-producing bacteria often prime the immune system to neu-

tralize the toxin of concern as soon as it appears in the body, thus preventing disease. Livestock in parts of the U.S. receive preparations consisting of *B. anthracis* cells that lack the protective capsule and thus replicate poorly. A similar vaccine for humans has been used in the former Soviet Union. But preparations that contain whole microbes often cause side effects, and they raise the specter that renegade cells might at times give rise to the very diseases they were meant to prevent.

AVA is given to soldiers and certain civilians but is problematic as a tool for shielding the general public against biological warfare. Supplies are limited. And even if AVA were available in abundance, it would be cumbersome to deliver on a large scale; the standard protocol calls for six shots delivered over 18 months followed by annual boosters. The vaccine has not been licensed for use in people already exposed to anthrax spores. But last year officials, worried that spores

Medical Lessons

Doctors now have a changed view of inhalation anthrax

By Ricki L. Rusting

THE RECENT CASES of inhalation anthrax in the U.S. have upended some old assumptions about that disease. When contaminated letters started appearing in September 2001, public health authorities initially believed that only those who received the letters, and perhaps individuals nearby, were in danger. But spores clearly seeped out through the weave of the envelopes, contaminating postal facilities and jumping to other mail. Such “cross contamination” is a leading explanation for the deaths of two of the 11 people confirmed to have contracted inhalation anthrax last year. Also contrary to expectations, spores do not remain sedentary once they land. They can become airborne again as people walk around in a tainted room.

One surprise was positive. Before October 2001 common wisdom held that inhalation anthrax was almost always incurable after symptoms appeared. But doctors beat those odds last fall, saving six of the victims. What made the difference? Researchers cannot draw firm conclusions from so few cases. But some intriguing patterns emerged when John A. Jernigan of the Centers for Disease Control and Prevention (CDC) and a team of others reviewed the medical records of the first 10 patients. Their findings appear in the November/December 2001 *Emerging Infectious Diseases* and online at www.cdc.gov/ncidod/eid/vol7no6/jernigan.htm

Relatively prompt diagnosis may have helped, the researchers report. Inhalation anthrax has two symptomatic phases—an early period marked by maladies common to a variety of ailments (such as fatigue, fever, aches and cough) and a later phase in which patients become critically ill with high fever, labored breathing and shock. Six of the 10 patients received antibiotics active against the anthrax bacterium, *Bacillus anthracis*, while they were still



NORMA WALLACE of Willingboro, N.J., is one of the six patients who survived inhalation anthrax last autumn.

showing early symptoms of infection, and only they survived.

The types of antibiotics prescribed and the use of combinations of drugs might also have had a hand in the unexpectedly high survival rate. Nine of the people discussed in the review sought care before the CDC published what it called “interim” guidelines for treating inhalation anthrax on October 26, but most patients received therapy consistent with those guidelines: ciprofloxacin (the now famous Cipro) or doxycycline plus one or two other agents known to inhibit replication of *B. anthracis* (such as rifampin, vancomycin, penicillin, ampicillin, chloramphenicol, imipenem, clindamycin and clarithromycin). Aggressive “supportive” care—including draining dangerous fluid from around the lungs—probably helped as well, scientists say.

Even the survivors were very sick, however. Jernigan says they are still being observed to see whether long-term complications will develop, although as of mid-January no obvious signs of such problems had emerged. Researchers suspect that anthrax antitoxins would ease the course of many people afflicted with anthrax and might also rescue patients who could not be saved with current therapies.

Ricki L. Rusting is a staff editor and writer.

might sometimes survive in the lungs for a long time, began offering an abbreviated, three-course dose on an experimental basis to postal workers and others who had already taken 60 days of precautionary antibiotics. People who accepted the offer were obliged to take antibiotics for an additional 40 days, after which the immunity stimulated by the vaccine would presumably be strong enough to provide adequate protection on its own.

In hopes of producing a more powerful, less cumbersome and faster-acting vaccine, many investigators are focusing on developing inoculants composed primarily of protective antigen produced by recombinant DNA technology. By coupling the recombinant protein with a potent new-generation adjuvant, scientists may be able to evoke good protective immunity relatively quickly with only one or

two injections. The dominant negative inhibitors discussed earlier as possible treatments could be useful forms of protective antigen to choose. Those molecules retain their ability to elicit immune responses. Hence, they could do double duty: disarming the anthrax toxin in the short run while building up immunity that will persist later on.

We have no doubt that the expanding research on the biology of *B. anthracis* and on possible therapies and vaccines will one day provide a range of effective anthrax treatments. We fervently hope that these efforts will mean that nobody will have to die from anthrax acquired either naturally or as a result of biological terrorism. SA

MORE TO EXPLORE

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Identification of the Cellular Receptor for Anthrax Toxin. Kenneth A. Bradley, Jeremy Mogridge, Michael Mourez, R. John Collier and John A. T. Young in *Nature*, Vol. 414, pages 225–229; November 8, 2001.

The U.S. Centers for Disease Control and Prevention maintain a Web site devoted to anthrax at www.cdc.gov/ncidod/dbmd/diseaseinfo/anthrax_g.htm