6th Annual Symposium
Giovanni Armenise-Harvard Foundation

June 27-29, 2002
St. Thomas, U.S. Virgin Islands

Structural Biology and Microbial Pathogenesis and the Host Response

About the Symposium

The threat of attack, the importance of defense, and the need to know more about each have become near universal worries since September 11. People who seldom thought about these issues in the wake of the Cold War now avidly follow the daily news. In contrast, attack, defense, and intelligence gathering are long-standing concerns for the select group of researchers who participated in "Structural Biology and Microbial Pathogenesis and the Host Response," the 6th Annual Symposium of the Giovanni Armenise-Harvard Foundation. Although the symposium's theme was decided before bioterrorism became a reality last fall, those events greatly magnified its relevance.

Anthrax, AIDS, tuberculosis, and malaria were among the globally important diseases explored in 22 invited lectures and an equal number of poster presentations. Investigators described using X-ray crystallography, as well as micro arrays and other post-genomic tools, to better understand the battle between pathogens and their hosts. The stakes are high: speakers emphasized that despite medical progress, infectious diseases still account for one in three deaths worldwide – the same toll they took on humankind 400 years ago.

The symposium was held June 27-29 at Marriott Frenchman's Reef, St. Thomas, U.S. Virgin Islands. Although the Caribbean is justly celebrated for its natural beauty, the people of these islands live with endemic malaria and tuberculosis, and this region is second only to sub-Saharan Africa in its rate of HIV infection.

In her closing remarks, Dr. Giulia De Lorenzo was optimistic about the impact of research presented during the symposium. New technologies such as structural biology, bioinformatics, and functional genomics will help generate not only new treatments and vaccines for combating human infectious disease, but also new strategies for protecting the global food supply. "Understanding the molecular logic of pathogenesis is crucial for future progress," said Dr. De Lorenzo, a professor of plant biology at the Universita' Di Roma La Sapienza and a member of the program committee for the symposium.

Last year, Foundation President and CEO Daniel C. Tosteson inaugurated a Career Development program that provides talented young Italian investigators with support needed to establish research laboratories in their home country. The 2002 Career Development awards were presented to Giampietro Schiavo and Rosella Visintin. Schiavo, who has been studying membrane dynamics at the nerve terminal at the Imperial Cancer Fund in London, will be joining the Department of Biological Chemistry at the University of Padova. Visintin has been

investigating key regulators of mitosis at the Massachusetts Institute of Technology, and will be returning to Italy to establish a lab at the European Institute of Oncology in Milano. Visintin was unable to attend the symposium.

Schiavo, who gave a lecture, was the only participant affiliated with a U.K. institution; 28 scientists came from five leading Italian research institutions and 48 represented the six basic science departments located on the Quadrangle at Harvard Medical School. Italian participants came from the European Institute of Oncology in Milano, the University of Padova, the Institute for Cancer Research and Treatment at the University of Torino School of Medicine, the Dipartmento di Ricerca Biologica e Tecnologica (DIBIT) at Scientific Institute San Raffaele in Milano, and Universita' Di Roma La Sapienza.

Presentations – Keynote Address

Reverse Vaccinology: a genome-based approach to vaccine development
Rino Rappuoli
IRIS, Chiron SpA, Siena

Historically, the first step in vaccine development was to grow the infectious agent in the laboratory. Once a pathogen was available in the lab, it was killed or attenuated for testing as a vaccine, those being the only approaches known prior to the advent of recombinant DNA technology. A new era began when genetic engineering made it possible for vaccinologists to scrutinize laboratory-grown organisms for individual antigens that might be used to elicit a protective immune response – a process that was labor-intensive and often quite slow.

Now, the ability to sequence the entire genomes of microorganisms has cleared a new path to vaccine discovery that Dr. Rappuoli called "reverse vaccinology." To illustrate how this new approach works, he described Chiron's work toward a preventive vaccine for group B meningococcus, a severe and sometimes fatal disease of children and adolescents. Instead of growing and manipulating large quantities of this pathogen, company researchers fed its genetic sequence into a computer programmed to scan for sequences that might encode antigens with potential as vaccines.

Meningococcus B challenged reverse vaccinology to demonstrate that it is not merely a new tool, but also a better one. After 40 years of trying, conventional methods had enabled vaccinologists to identify between 15 and 20 potential group B meningococcus antigens. Unfortunately, tests

showed that none of these provided universal protection against disease. When Chiron scientists fed the organism's full sequence into the computer, it predicted that some 600 antigens encoded by the bacterium might have promise for vaccine development. These were expressed as recombinant proteins in *Escherichia coli* and nearly 350 were tested in mice; about 90 were novel surface proteins and 29 of these appeared to stimulate desirable immune responses. Within 18 months, reverse vaccinology had yielded more vaccine candidates than traditional approaches generated in four decades, Dr. Rappuoli noted. The best of these novel antigens have been incorporated into what developers hope will be a universally protective vaccine, a candidate that recently entered Phase I clinical trials.

Chiron is now applying reverse vaccinology to the genomes of other bacterial pathogens, including pneumococcus, streptococcus, staphylococcus and the organisms responsible for malaria and tuberculosis. Genome-based predictive technology is also being used to design vaccines against HIV and other viruses.

Dr. Rappuoli characterized the AIDS pandemic as "today's Black Plague," and warned that unless the economics of vaccine development can be changed, there will be little incentive for companies to develop vaccines against AIDS and other diseases that mostly kill people in the developing world. Although vaccines are the most cost effective medical intervention ever known, they are less financially attractive to companies than therapeutic drugs. He ended his lecture with a plea that rich nations shoulder more responsibility for making vaccines that are desperately needed around the world.

Anthrax toxin and ways to inhibit it
R. John Collier
Department of Microbiology and Molecular Genetics, Harvard Medical School

The events of fall 2001 galvanized public interest in bioterrorism, and added the term "weaponized" to the vocabulary of millions. Although there is no generic defense against pathogens that can be turned into weapons, Dr. Collier is confident that basic research will yield strategies for blunting the lethal power of anthrax, the microbe that his laboratory has studied for 15 years.

As most newspaper readers became aware last fall, people who come into skin contact with the anthrax bacterium are very likely to survive infection, whereas infection is nearly always fatal in people with the misfortune to inhale it. At present, the only defense is a limited supply of a not very good vaccine; new and better vaccines won't be available for at least several years, and

there is as yet no public health strategy for mass immunization. As a result, Dr. Collier said, the nation is undeniably vulnerable to anthrax attack. This vulnerability can be reduced, however, if better scientific insights into anthrax virulence can be translated into defensive stratagems.

Bacillus anthracis produces its toxin in three parts: Protective Antigen (PA) binds to a receptor on the host cell and facilitates entry of two enzyme effectors known as Edema Factor (EF) and Lethal Factor (LF). As their names suggest, EF causes swelling whereas LF causes death. Originally secreted from the bacteria as nontoxic monomers, or small molecular building blocks, these proteins assemble on the surface of receptor-bearing cells to form toxic noncovalent complexes. PA binds to cells, coordinates self-assembly of the complexes, and ultimately delivers EF and LF to the cytosol. EF is a calmodulin-dependent adenylate cyclase, and LF is a zinc metalloprotease that cleaves members of the MAPKK family and possibly other intracellular substrates.

New findings from Dr. Collier's lab and other research teams indicate that after PA complexes with EF and LF on the cell surface, the resulting structures change shape several times as they insinuate themselves through the membrane and into the cell. A fuller understanding of these structure-function relationships suggests three possible ways to abort their deadly mission, Dr. Collier reported. The first is a dominant-negative inhibitor, a slightly mutated form of PA that appears to protect injected rats against exposure to anthrax toxin. Another possibility is a polyvalent inhibitor, a small peptide that partially blocks LF binding with PA on the cell surface; it is also being tested in animals. Finally, it may be possible to make a soluble form of the PA receptor that would attach itself to the anthrax toxin before it reached host cells. All three are under active study.

Tetanus toxin and its journey in motor neurons
Giampetro Schiavo
Molecular Neuropathobiology Laboratory, Cancer Research UK, London Research Institute

Infections caused by members of the *Clostridia* family have long fascinated neuroscientists, in part because neurotoxins from related bacteria use shared cellular pathways to produce dramatically different clinical effects. *C. tetani* causes tetanus, marked by lockjaw, the fixed smile known as *risus sardonicus*, and rigid spastic paralysis that can be fatal. *C. botulinum*, in contrast, causes widespread muscle weakness that at worse becomes a flaccid paralysis that can kill. Neither is something that a doctor wants to discover in a patient. In the laboratory, however,

neurobiologists have used both types of neurotoxins as tools for understanding transport mechanisms in nerve cells.

Dr. Schiavo focuses on axonal retrograde transport, a mechanism necessary for neuronal survival, in which neurotropins are transported from nerve terminals to the cell body. The distances involved in axonal transport can be impressive: in human terms, the distance from a giraffe's brain to its spinal motor neurons would be twice the distance of the Tour de France.

Organelles, pathogens, neurotrophins and other synaptic signaling molecules use similar mechanisms to journey from nerve ending to cell body. Tetanus toxin (TeNT) is among the virulence factors that make this trip, which begins when it binds at the neuromuscular junction and eventually leads to the spinal cord. There, TeNT's interference with the normal release of inhibitory neurotransmitter results in spasticity that is the hallmark of tetanus infection.

Although scientists know quite a bit about the intracellular catalytic activity of TeNT, control of its retrograde transport is less well understood. Dr. Schiavo's team invented a transport assay that uses a nontoxic TeNT fragment (TeNT H_C) as a probe. Working with living motor neurons, they determined that TeNT binds to a structure that forms only in lipid-rich microdomains in the cell membrane, and they soon realized that sequestering cholesterol could block entry of TeNT. When they permitted TeNT H_C to bind, it was rapidly endocytosed and transported in two types of vesicular carriers, one round and one tubular, which travel at different speeds using microtubules and the actin cytoskeleton. The organelles are not acidified during axonal transport and lack typical endocytic markers. Dr. Schiavo's team discovered that one of these compartments is the normal one for transporting nerve growth factor (NGF), which is apparently hijacked by TeNT H_C for retrograde axonal transport. When Dr. Schiavo moves to the University of Padova, he and collaborators there will use a proteomics approach to characterize these transport compartments more precisely.

Genetic differences in the susceptibility of the host to anthrax lethal toxin
William F. Dietrich
Department of Genetics, Harvard Medical School

There are two sides to the story of every infectious disease, one told by the pathogen and the other by the host, and Dr. Dietrich has been listening closely to the host. The Lethal Factor (LF) component of anthrax toxin kills macrophages, key players in innate and acquired immunity, by rupturing their plasma membranes and causing the cell's contents to spill. Although LF is known to be a zinc metalloprotease that cleaves members of the MAP (membrane-associated protein)

kinase family, exactly how these cleavage events lead to the death of macrophages is unclear. Dr. Dietrich's laboratory has been exploring this question using inbred mouse strains whose macrophages exhibit striking differences in susceptibility to the effects of LF. These differences have been traced to a single gene, located downstream from toxin entry, called *Ltxs1* (lethal toxin sensitivity 1).

Ltxs1 has been mapped to mouse chromosome 11, and Dr. Dietrich's team has determined that it is actually the same gene as *Kif1C*, which encodes a kinesin-like protein involved in microtubule transport of an unknown cargo. Several lines of evidence support the idea that the two are identical. First, *Kif1C* is the only gene in the *Ltxs1* interval exhibiting polymorphisms between susceptible and resistant strains. Second, multiple alleles of *Kif1C* determine susceptibility or resistance of cultured mouse macrophages to intoxication with LF. Finally, treatment of resistant macrophages with Brefeldin-A (which disrupts Kif1C localization) induces susceptibility to LF intoxication, while ectopic expression of a resistance allele of *Kif1C* in susceptible macrophages causes a 4-fold increase in the number of cells surviving LF intoxication. With these observations in hand, Dr. Dietrich said the next steps in his research will involve working out the cell biology of *Kif1C* to discover where it is localized and exactly how it operates.

Studies on the involvement of SNARE proteins in endosomal vacuolization induced by *Helicobacter pylori* VacA toxin
Marina de Bernard
Dipartimento di Scienze Biomediche Sperimentali, University of Padova

Helicobacter pylori, a Gram-negative bacterium involved in the development of chronic gastritis, peptic ulcer and gastric lymphoma, produces a toxin, called VacA, which induces vacuole formation in epithelial cells in culture. These vacuoles originate from late endocytic compartments, as demonstrated by the presence of the small GTPase Rab7 in their limiting membrane. The presence of a Rab protein signifies that a SNARE protein on the endosomal transport compartment has been properly matched with a corresponding SNARE protein on the target membrane.

Recent studies show that after VacA binds, it undergoes conformational changes and forms an anion-specific channel that the late endosomal compartment passes through before ballooning into a vacuole that may be 400 times larger than the original compartment. This dramatic size increase caused Dr. de Bernard to ask whether the membrane from a single original compartment provides enough raw material for such a huge expansion, or whether additional material must be gathered from other vesicles. If other materials are required, they may derive from several

sources: homotypic fusion of late endosomes, fusion of endosomes and lysosomes, or deposition into the membrane of carrier vesicles derived from early endosomes. Each of these fusions hinges on a SNARE hook-up that cannot take place without a cytosolic protein called α-SNAP.

Dr. de Bernard's team transfected cells with a dominant negative mutant of α-SNAP, then added VacA extracellularly or expressed it within the cell. If vacuolization was dependent on SNARE-mediated fusion, then no vacuoles were expected to form. In fact, the investigators observed that VacA had no difficulty inducing huge, swollen vacuoles even though SNARE-dependent fusion had been blocked. This finding led Dr. de Bernard to hypothesize that raw materials for vacuole enlargement are already present in the original endosomal compartment, and that Rab7 may be the key that turns internal membrane vesicles (or invaginations) into an outer membrane for the vacuole. Support comes from recent experiments showing that cells transfected with a dominant-negative Rab7 mutant did not form vacuoles when exposed to VacA, and further investigations are underway.

Clostridial neurotoxins: mechanism of action and therapeutic use
Cesare Montecucco
Centro CNR Biomembrane and Dipartimento di Scienze Biomediche, University of Padova

Clostridial neurotoxins comprise one tetanus neurotoxin (TeNT) and seven botulinum neurotoxins (BoNT), which cause tetanus and botulism, the contrasting neuroparalytic syndromes that Dr. Schiavo described in his lecture (above). Dr. Montecucco's laboratory conducts structure-function studies of these neurotoxins, and his findings relate both to botulism as clinical disease and to BoNT's role as an increasingly popular therapeutic agent.

The crystallographic structures of BoNT/A and BoNT/B show the presence of a first shell of coordinating residues (2 histidines and 2 glutamic acids), and a second shell of residues close to the catalytic center, which are different from any known metalloprotease and which are thought to explain the structures' unique spectroscopic properties. When Dr. Montecucco's team mutated several second shell residues in TeNT and BoNT/A, they found parallels between the spectroscopic and catalytic properties of the mutant neurotoxins. They also saw that the metalloproteolytic activity of these neurotoxins is strongly activated by lipids that resemble those in synaptic vesicles, a finding consistent with Dr. Schiavo's observation that TeNT needs cholesterol to enter cells.

Physicians have long observed that patients who survive the acute phase of botulism, when paralysis affects respiratory muscles, are likely to make a good recovery. Similarly, clinicians

who use BoNTs to treat "everything from stroke to writer's cramp" know that the benefits don't last forever. In a series of experiments with rats, Dr. Montecucco found that damage caused by introducing BoNT/A at the neuromuscular junction diminishes as nerve terminals generate new sprouts over time. BoNT/A is the toxin approved for clinical use, and these animal experiments help explain why repeated injections are necessary. Other types of botulinum toxins may work better. Dr. Montecucco and his clinical collaborators have shown that BoNT/C provides more durable relief for several dystonias of facial muscles, and they are now evaluating the therapeutic potential of other BoNT serotypes as well.

Comprehensive identification of *Mycobacterium tuberculosis* genes required for infection

Eric J. Rubin
Department of Immunology and Infectious Diseases, Harvard School of Public Health

When the first widespread epidemics of tuberculosis struck Europe in the 16th Century, the disease swept through all levels of society. As living conditions improved for the middle and upper classes, however, the disease settled among the urban poor and the people of underdeveloped countries. More recently, the AIDS pandemic has given TB a tremendous boost, many people have been inadequately medicated, and ferocious drug-resistant strains have surfaced in far-flung parts of the globe. Despite TB's status as one of the world's leading causes of death, less is known about it at the molecular level than about other pathogens that kill far fewer people. One reason for this relative lack of information, Dr. Rubin said, is that pharmaceutical companies do not perceive the developing world as a profitable marketplace for drugs.

The function of thousands of genes in the *M. tuberculosis* genome remains unknown. To address this problem, his laboratory has invented a novel technique, transposon site hybridization (TraSH), which can be combined with micro array technology to identify all the genes expressed in specific growth conditions. Dr. Rubin's team is using this approach to determine the full set of genes needed by *M. tuberculosis* to cause disease.

Transposons are naturally occurring, mobile genetic elements whose movements can be manipulated in the laboratory. TraSH combines high-density transposon mutagenesis with micro array mapping of insertion sites in pools of mutants. So far, about 100,000 mycobacteria mutants have been created using a *mariner*-based transposon and efficient phage transduction. TraSH has enabled Dr. Rubin and his colleagues to define specific sets of genes required for the survival of pathogenic mycobacteria grown under a variety of different

conditions. This technique has allowed them to identify genes that *M. tuberculosis* needs to proceed through progressive stages of infection in a mouse model. Not surprisingly, the genes required early in the infection differ from those needed later on. At first, a relatively small number of genes are essential – most of them needed to acquire nutrients. But as infection progresses, and the host fights back, the pathogen requires an increasing number of genes to survive. After 8 weeks of infection, 173 essential genes were identified, and scientists have yet to identify exactly what 107 of them do. Having so many genes in play at once, however, may explain why the TB pathogen is so adept at developing resistance to treatment.

The logic of biosynthesis of the glycopeptide antibiotic vancomycin
Christopher Walsh
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School

Vancomycin was approved by the Food and Drug Administration in 1958, but did not come into widespread use until the 1980s. It soon gained a reputation among physicians as the antibiotic of last resort for severe infections involving Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis*, and its use undoubtedly saved many lives. Reports of vancomycin-resistant *Enterococcus* began to surface as early as 1988, however, and more recently these have skyrocketed. Scientists now know that a single biochemical change in the bacterium can confer as much as a thousand-fold increase in resistance, Dr. Walsh said.

There is a pressing need to re-engineer vancomycin into a new antibiotic that will kill drug-resistant bacteria. And the way to do so, according to Dr. Walsh, is to examine how *Actinomycetes*, a family of filamentous bacteria, originally designed vancomycin as a weapon to wield against their enemies. If the creator's original logic is understood, then science will know where to go next. The scientists are like a team of industrial engineers, analyzing every step in an automobile assembly line to learn how it can be retooled for next year's model.

Earlier studies showed that peptide antibiotics such as vancomycin, penicillins, and pristinamycins are biosynthesized by nonribosomal peptide synthetases (NRPS) acting as multimodular protein assembly lines. The manufacturing genes for these nonribosomal peptides are clustered together, presumably so they can regulate and coordinate one another's activities. Dr. Walsh's team has found that vancomycin production is driven by a cluster of 30 genes in the organism *Amycolocaptosus orientalis.* These genes encode enzymes that make amino acid monomers. A three-subunit NRPS assembles these monomers, along with deoxyhexose

vancosamine, into the heptapeptide backbone of the antibiotic. This acyclic heptapeptide is then oxidatively crosslinked by tandem action of three hemeproteins, then glycosylated by specific glycosyl transferases to create vancomycin. Now that Dr. Walsh and his colleagues have characterized the enzymatic steps needed to build vancomycin, the next step is to reprogram the assembly line and synthesize novel antibiotics that will side-step the defenses of resistant pathogens.

Host-pathogen recognition: the structural basis of the interaction between fungal polygalacturonase and its plant inhibitor PGIP
Benedetta Mattei
Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza"

When a fungus initially attacks a plant, it must batter its way through the cell wall while the plant struggles to resist. A series of studies at the Universita di Roma La Sapienza have gradually uncovered the battle that rages between fungal proteins, called polygalacturonases (PGs), and corresponding plant recognition proteins, known as polygalacturonase-inhibiting proteins (PGIPs). As part of this ongoing work, Dr. Mattei and her colleagues use X-ray crystallography, augmented with a variety of biophysical and biochemical techniques, to perform structural analysis of interactions between specific PGs and their corresponding PGIPs,

In the cell wall, one of the first steps toward disease is for PGs to hydrolyze homogalacturonan. Fighting back are PGIPs, which Dr. Mattei and her colleagues previously found to comprise 10 leucine rich repeats (LRRs) – motifs specialized for interaction with PG. Two years ago, her group solved the crystal structure of PG from the phytopathogenic fungus *Fusarium moniliforme*, describing it as a right-handed parallel β-helix with 10 coils, each made up of three or four parallel β-helical strands. Overall, it resembles a squared-off coil of spring. These findings led to more questions about what happens when PG and PGIP interact.

Her recent experiments used surface plasmon resonance and mass spectrometry to characterize the PG-PGIP interface, as well as site-directed mutagenesis to locate the PG residues involved in catalysis and some of the residues recognized by PGIP. By cloning and characterizing PGIPs from Arabadopsis and bean plants, they have learned that a single amino acid difference can determine whether or not a PGIP inhibits *F. moniliforme* PG. They have also learned that indigenous plant PGs, needed for normal processes, differ slightly from fungal PGs and are able to go about their business without being recognized and attacked by PGIPs. When a PGIP inhibits the activity of its corresponding fungal PG, it appears to do so by competing with the substrate for binding sites and covering the active site cleft, Dr. Mattei said. She predicted that

structure-function studies now underway in her laboratory, including a recent crystal structure for PGIP from *Phaseolus vulgaris,* will shed additional light on how plants protect themselves against enemies.

Mechanisms of drug resistance in protozoan parasites: a window into parasite evolution
Dyann Wirth
Department of Immunology and Infectious Diseases, Harvard School of Public Health

Four hundred years ago, infectious diseases accounted for one third of global deaths, and the same is true today, Dr. Wirth said. In some cases, decades of medical progress have been reversed as organisms developed resistance to drugs. Malaria is a vexing example: *Plasmodium falciparum* needed 16 years to develop strains resistant to chloroquine, but only 4 years to begin outsmarting fansidar and another 4 years for mefloquine resistance to surface. Strains that foiled the newest anti-malarian drug, atovaquone, showed up after only 6 months.

Drug resistance in malaria and other pathogens has been traced to over-expression of carrier proteins from the ABC transporter superfamily, which are typically seated in the cell membrane and serve to pump drugs out of the organism. Dr. Wirth's team has been investigating resistance mechanisms mediated by ABC-transporters in two protozoan parasites, *P. falciparum* and *Leishmania enriettii*. In a *Leishmania enriettii* model, vinblastin resistance is controlled by a single gene, LeMDRI1. Localization experiments indicate that this gene is expressed in the flagellar pocket and other vesicular compartments, rather than on the surface of the parasite. Dr. Wirth proposes a multi-step model for resistance that involves drug sequestration and subsequent vesicular transport. Initially, expression of the ABC transporter increases in response to drug treatment. Parasites with the highest levels of ABC transporter proteins have a survival advantage, and go on to develop specific point mutations within the transporter gene that confer resistance. When Dr. Wirth and her colleagues compared laboratory strains of drug-resistant *P. falciparum* with isolates from patients who were failing treatment in the community, point mutations were more common in the primary isolates.

These observations led them to a broader investigation of specific point mutations and single nucleotide polymorphisms within *P. falciparum*. So far, most of what is known about the genetics of survival in this globally important parasite comes from examining one gene at a time. The *P. falciparum* genome is nearly complete, however, and when it is finished the next step will be using genomics to discover all the players in drug resistance. So far, Dr. Wirth's analysis shows far less genetic variation in *P. falciparum* than expected, which suggests that it is a relatively recent scourge – perhaps only about 10,000 years old. Resistance and pathogenicity

genes display more polymorphisms than other parts of the genome, however, which probably helps them evade attack by drugs or host immune responses. If more of these advantageous polymorphisms can be identified, some may turn out to be new drug and vaccine targets in *P. falciparum*.

Polygalacturonase-inhibiting proteins in plant defense against phytopathogenic fungi
Giulia De Lorenzo
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Like an invading force that arrives by land, sea, and air, pathogens attack plants with many weapons at once. That being the case, it's a good thing that plants have what Dr. De Lorenzo calls "impressive redundancy in their defensive arsenal." Central to the battle between fungus and plant is the recognition system introduced earlier in the symposium by Dr. Mattei: polygalacturonase-inhibiting proteins (PGIPs) in the plant's cell wall recognize fungal endopolygalacturonases (PGs), move to limit local fungal damage, and form pectic fragments that initiate a chain of defensive responses.

Dr. De Lorenzo and her colleagues have done extensive research on PGIPs. Early on, they realized that the versatile leucine-rich repeat (LRR) structure of these cell wall proteins suggested that plants might generate a PGIP for every PG secreted by their fungal enemies. This echoes the human immune system's capacity for producing antibodies that recognize a vast array of pathogens. Just as antibodies are only one component of the human immune response, Dr. Lorenzo and other investigators have found that recognition of pathogenicity factors is the first of several steps toward disease resistance, and that plants vary in their capacity to identify and resist specific invaders.

Her lab is now exploring how basic science insights can be used to improve the global food supply. Possible strategies for strengthening plant disease resistance include making PGIPs more alert sentinels, reprogramming defensive pathways, or modifying the defense response itself. This might involve transforming crop plants with different *pgip* genes. In Africa, for example, stored crop seeds are often poisoned and made useless by the fungus *Fusarium moniliforme*. Some PGIPs from bean plants are exquisitely sensitive to PGs from *F. Moniliforme*, and the genes for these watchdog proteins might be transferable to susceptible plants. In addition to sharpening the plant's ability to recognize PGs deployed by its enemies, Dr. De Lorenzo's group is also investigating how multiple defenses might be mobilized when pathogens strike with several weapons at once.

Recognition of bacterial effector proteins by the plant innate immune system
Brian Staskawicz
Department of Plant and Microbial Biology, University of California at Berkeley

Building on the themes of Dr. De Lorenzo's lecture, Dr. Staskawicz described how post-genome technology is being used to create disease-resistant plants. In his laboratory, experiments are done using a model system where the genome sequences of both plant and pest are known. *Pseudomonas syringae* is a Gram-negative organism that causes bacterial speck disease on *Arabidopsis thaliana*, a favorite model plant. Because both genome sequences are available, a bioinformatics approach can be used to identify putative effector proteins in *P. syringae.*

Like other pseudomonads, this plant bacterium is equipped with a Type III secretion system that injects effector proteins inside host cells, where they cause disease. The products of resistance genes can defuse these time bombs if they spot them right away and kill cells around the infection site, which keeps damage from spreading. Dr. Staskawicz' team has identified arabidopsis resistance genes that appear to be part of a toll-like receptor (TLR) system. TLRs are a highly conserved part of innate immune systems in simple animal models, such as fruit flies, and in mammals and humans as well. If scientists know the genes involved in these pathways, it should be possible to use these to block proteins encoded by specific genes in the pathogen. The value of this gene-gene resistance strategy is now being tested by collaborators of Dr. Staskawicz at the University of Florida. A gene that makes pepper plants resistant to bacterial spot disease has been inserted into tomato plants, which are now being grown in the field. So far, Dr. Staskawicz says that the transgenic plants appear to suppress this common disease quite well.

Plant defense responses against necrotrophic fungi are activated through multiple signaling pathways.
Simone Ferrari
Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School

Dr. Ferrari began his talk by noting that about 15% of all agricultural crops are lost to disease, which puts the annual global cost of plant infections at about $500 billion per year. Fungi pose a major threat to crops, which is why laboratories like his are extremely interested in characterizing defense responses that can fend off specific types of these pathogens. Fungi can be divided into two broad categories: biotrophic ones that grow only on the living tissues of specific plants, and necrotrophic pathogens that kill host cells and can colonize a wide range of hosts. The necrotrophic fungi macerate plant tissues with degrading enzymes such as

polygalacturonases (PGs), which were the topic of several other presentations during this symposium.

Dr. Ferrari's lab studies the interaction between the model plant *Arabidopsis thaliana* and the necrotrophic fungus *Botrytis cinerea*, a pre- and post-harvest pathogen that is to blame for many of the rotting vegetables found in home refrigerators. It infects more than 200 plants, and is difficult to control because pesticides obviously can't be used on plants harvested as foods. As an alternative to agricultural poisons, his lab studies how selective activation of defensive responses might protect against this pest.

In general, plants use three signaling pathways to defend against fungi: ethylene (ET), jasmonate (JA), and salicylic acid (SA). Necrotrophic fungi such as *B. cinerea* activate two of these paths, ET and JA, Dr. Ferrari said. His team has recently characterized two Arabidopsis genes that encode polygalacturonase inhibitors (PGIPs) effective against PGs from *B. cinerea*. Although over expression of either gene reduces disease symptoms in Arabidopsis, Dr. Ferrari has found that during infection the protective genes AtPGIP1 and AtPGIP2 are called into battle by separate signals – much as Army and Navy forces respond to different commanders. The JA pathway uses one set of signaling molecules to activate AtPGIP2, whereas different intermediaries turn on AtPGIP1. The next step will see whether inducing multiple defense responses through separate pathways may be an effective way to protect plants against versatile, wide-ranging fungi such as *B. cinerea.*

Genome science in bacterial pathogen biology and evolution
John J. Mekalanos
Department of Microbiology and Molecular Genetics, Harvard Medical School

Nearly 70 bacterial genomes have been fully sequenced and another 160 are in progress, a development that is revolutionizing the study of microbial pathogens. Once an organism's complete genome sequence is available, researchers can learn much about its biology by using sequence homology to assign or guess at the functions of its genes. In addition, various post-genome technologies enable researchers to analyze extremely large numbers of genes simultaneously. In Dr. Mekalanos' lab, these new methods have yielded important insights into the evolution of *V. cholerae* strains responsible for a series of cholera pandemics, as well as details about the molecular basis for *V. cholerae* pathogenesis.

Working with collaborators at The Institute for Genetics Research, Dr. Mekalanos' team sequenced a *V. cholerae* strain called N16961. They developed micro arrays containing at least

93% of all open predicted reading frames (potential genes) for this strain, and used these arrays to compare the genomes of several *V. cholerae* strains. They found a set of genes common to all pandemic biotypes, as well as genes that set the so-called classical strain, which is blamed for six earlier pandemics, apart from its successor, the 7th pandemic or El Tor biotype. Only 22 genes differentiate El Tor from the classical strain and from other strains that fizzled before causing global disease; now the challenge is to determine exactly which gene products make the El Tor strain such a threat to public health.

Already Dr. Mekalanos' team has identified several genes that make the El Tor strain what it is and which might be good antibiotic targets because their proteins are essential for bacterial replication. Another project has focused on quorum sensing, the mechanism that enables bacteria colonizing the intestinal tract to sense and regulate their own density. This population control measure might be used against the microbes, if a drug could signal "enough bacteria already" when in fact there are only a few. One of the quorum sensing genes also appears to help regulate expression of important *V. cholerae* virulence genes. The investigators are now profiling *V. cholerae* gene expression in a range of animal and human hosts as well as different laboratory growth conditions, with the goal of learning more about how *V. cholerae* senses and responds to host signals.

Eventually, Dr. Mekalanos hopes these investigations will contribute to better treatments, a preventive vaccine, and environmental control strategies that can prevent thousands of cases at once, rather than combating cholera one patient at a time.

Searching for the function of *Neisseria meningitidis* B proteins identified on a genomic base
Emanuele Papini
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For the most part, the Gram-negative bacteria *N. meningitidis* dwells without incident in the human nose and throat. Every so often, however, it invades the epithelium and diffuses into the bloodstream. When this happens, "no other infection so quickly slays," in the words of an early 20th Century physician. Meningitis or fatal meningococcal sepsis is especially likely in children, and although the survival rate is about 90% for cases identified early, the treatment window is very narrow and outcomes are especially poor when diagnosis is delayed.

Although preventive vaccines are available for several pathogenic serotypes of *N. meningitides*, none has been developed for serogroup B. By analyzing the genome of the MC58 strain of *N. meningitidis* B, Dr. Papini and his colleagues have identified potential virulence factors including

extracellular proteins that interact with host mucosa and blood. They have used *E. coli* to express highly purified preparations of these proteins, some of which appear promising for vaccine development. So far, the most interesting of these is a lollypop-shaped protein on the *N. meningitidis* surface called NadA, which is endocytosed by human monocytes and can act at the level of the bloodstream as well as at the mucosa. In the laboratory, it has a high affinity for mucosa-like Chang cells, suggesting that it is an adhesion factor. NadA also triggers release of cytokines that might initiate or worsen septic shock in patients. But the most interesting possibility, which Dr. Papini's lab is currently pursuing, is that it might be a novel vaccine candidate.

Structural and biochemical characterization of the poliovirus cell entry pathway.
James M. Hogle
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School

More than 15 years ago, Dr. Hogle determined the atomic structure of poliovirus. Since then, his research has revealed that when the virus enters a target cell, it resembles a Mars landing module that settles into place, drills into the surface, then pours genetic material into the hole. Structural, kinetic, biochemical, and genetic studies all contributed to this understanding of how the virus breaches cells.

The drama begins in the extracellular environment: as the mature virion travels from cell to cell or host to host, a maturation cleavage of a capsid protein, VP0, locks it in a meta-stable state. The virus remains rigidly stable until it encounters Vpr, its matching receptor on target cells. Upon contact, the receptor acts as a catalyst that overcomes an energy barrier and frees up the meta-stable state. A series of conformational changes, which Dr. Hogel's team has documented over the years, allows the virus to attach to membranes, form a pore, and release its RNA genome into the cytoplasm. This sequence of events appears to be a prototype for the maturation and cell entry of more complex enveloped viruses, such as influenza and HIV.

Now that they have the big picture, Dr. Hogle and his colleagues have developed a simple, liposome-based model system for carrying out detailed structural and biochemical studies of poliovirus' entry into membranes. Liposomes decorated with poliovirus receptors are used to capture the virus, then purified for electron microscopy. The researchers have demonstrated that receptor-decorated liposomes induce conformational changes that enable poliovirus to attach directly to membranes, and preliminary data suggest that the Pv-decorated liposomes allow RNA translocation. These studies are ongoing.

Dr. Hogle introduced his presentation by noting that his scientific career has been devoted to studying a pathogen which, if the World Health Organization's 2005 eradication goal is met, will soon be eliminated. He eagerly anticipates this moment, and remarked that he always knew that eventually the urgency for studying this organism would diminish.

Under construction: building a reovirus factory from viral and cellular components
Max L. Nibert
Department of Microbiology and Molecular Genetics, Harvard Medical School

The family *Reoviridae* includes genera whose members infect plants, invertebrates, and vertebrates and cause disease in non-human hosts ranging from fish to horses. The reoviruses of greatest clinical importance in humans are the group B rotaviruses, which cause diarrheal disease. Reovirus-infected cells are distinguished by the presence of structures called viral inclusions, which in some cases serve as factories where the viral RNA genome is replicated and large, non-enveloped viral particles are built.

Much as engineers might analyze the workings of an automobile factory, Dr. Nibert and his colleagues set out to determine how these virus factories are formed, where raw materials come from, and how they are organized. The purpose of this investigation is to figure out how antiviral drugs might someday disrupt this viral assembly line. The output of these factories is a non-enveloped virus with two capsid proteins, 10 genomic segments, a double strand of linear RNA in its core, and a well-defined three-dimensional structure. A reovirus is partly disassembled upon cell entry, genomic segments are transcribed in the cytoplasm, and the resulting proteins are fed into the factory.

Dr. Nibert hypothesized that something as neatly constructed as a reovirus must be built in a highly ordered intracellular environment. By using immunofluorescence microscopy and a panel of antibodies to viral and cellular proteins, his team found that reovirus factories come in two shapes, globular and filamentous, with the filamentous shape prevailing in 22 of 24 strains tested. They determined that this shape occurs when the factories are associated with cellular microtubules, a linkage accomplished by a reovirus core protein called μ2, an NTPase involved in viral RNA synthesis. The role of μ2 was confirmed when the investigators introduced a point mutation and the factories became globular. Dr. Nibert's group has since studied how raw materials for virion construction are recruited into the factories. Experiments show that μ2 also plays a role here, by recruiting a major reovirus nonstructural protein, μNS, thought to bind RNA, to filamentous factories. Current studies are addressing other protein–protein and protein–

RNA interactions, as well as considering how components of the cellular ubiquitin–proteasome system are associated with the factories.

Down-regulation of MHC class I molecules by human cytomegalovirus
Domenico Tortorella
Department of Pathology, Harvard Medical School

Major histocompatibility complex (MHC) molecules play an important role in human immunity by restricting T-cell responses to protein antigens. A key function of MHC class I molecules is to bind peptides generated by invading viruses or bacteria, and to present them on the surface of infected cells so that CD8+ T cells will attack and destroy them. Every step in the formation of MHC-antigen complexes is vulnerable to interference, and Dr. Tortorella has been studying a strategy that human cytomegalovirus (HCMV) uses to thwart this antigen presentation system. The evasion strategy used by this virus has broad medical implications because it parallels events that occur in cystic fibrosis, and could point the way to better treatments for this severe, inherited disorder.

HCMV generates two unique gene products, US2 and US11, that subvert normal steps in MHC class I antigen presentation. For starters, US2 and US11 speed the breakdown of class I heavy chains, which interferes with antigen binding by the transmembrane tail of the MHC molecule. The heavy chains are extracted from the endoplasmic reticulum (ER) and relocated to the cytosol, where they are shuttled to the proteasome – the cellular equivalent of a garbage disposal – where they are ground up and thrown away. This is a normal waste removal method that cells use to rid themselves of misfolded proteins and other trash, Dr. Tortorella noted, which US2 exploits by tagging normal MHC class I molecules for premature degradation. The breakdown of class I heavy chains is strikingly similar to the degradation of a mutant form of cystic fibrosis transmembrane conductance regulator (CFTR) which has been implicated in progression of CF. Both seem to be four-step processes, Dr. Tortorella said, involving recognition, recruitment, relocation, and degradation.

His group is using several methods to shed light on additional functions of US2 and US11. A mutagenesis study using deletion and chimeric mutants of US2, US11 and class I heavy chains suggests that the cell employs different complexes to extract proteins from the ER. An in vitro degradation system was used to identify ER and cytosolic proteins involved marking MHC class I molecules for destruction. More recently, high-throughput screening was used

to examine more than 16,000 chemicals to see if any would interfere with US2- and US11-mediated destruction of class I heavy chains. Four inhibitors were identified, Dr. Tortorella said, and he is certain these will be valuable tools for studying the degradation reaction. It is even possible that one of these may eventually turn out to be a viable drug for cystic fibrosis or other diseases that involve degradation of misfolded, but potentially functional proteins.

Three-dimensional structure of iron-binding proteins from *Helicobacter pylori* and *Bacillus anthracis*

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At first glance, one might not expect a high-profile biological warfare agent and the leading cause of ulcers to have much in common. What they turn out to share, however, is that both need iron to grow properly. *Bacillus anthracis* has been in the news since it was deployed as a bioterrorism agent last fall, and there is intense interest in developing novel, second-generation preventive vaccines that are safer than the current one, which is notorious for its adverse effects. The path to a new kind of vaccine might be illuminated by comparisons of anthrax's iron-binding proteins with those found in *Helicobacter pylori*.

In the *B. anthracis* genome, Dr. Zanotti and his colleagues have identified two genes that encode ferritins, proteins that store iron and reduce oxidative stress. Called Dlp-1 and -2, these are dodecamers homologous to the DNA binding protein of *Escherichia coli*, called Dps. Dps-like proteins are generally highly immunogenic. *Helicobacter pylori,* which is best known for its association with ulcer disease, has an iron-binding protein known as HP-NAP, which has the unusual ability to activate neutrophils. Because it has proved highly immunogenic in humans and mice, it has been incorporated into an experimental *H. pylori* vaccine being developed by Chiron, Dr. Zanotti noted.

His lab has recently solved the crystal structures of Dlp-1, Dlp-2 and HP-NAP grown in *E. coli*. Dr. Zanotti's team showed that each has a quaternary structure similar to that of the dodecameric bacterial ferritins in the Dps-like family: they are sphere-like proteins with 32-facets and an internal cavity. Functional studies confirm that Dlp-1 and Dlp-2 are involved in iron uptake and regulation, fundamental activities during bacterial growth. HP-NAP has a different distribution of surface-potential charges, due to the presence of many positively charged residues, and Dr. Zanotti speculates that this may explain why it can activate human leukocytes while Dlp-1 and -2 cannot. HP-NAP is a promising vaccine component, and although it has features that Dlp-1 and -

2 do not, nevertheless the similarities among these ferritins may auger well for development of future anthrax vaccines.

Bacterial/cytolysin-mediated delivery of protein to the cytosol of mammalian cells. Darren Higgins
Department of Microbiology and Molecular Genetics, Harvard Medical School

Listeria monocytogenes is a common bacterium that preys mainly on infants, elderly people, and pregnant women. It is among the most lethal of food-borne pathogens, with a case fatality rate of 25% to 60%. Because it has ingenious mechanisms for getting into mammalian cells and spreading from one cell to the next, Dr. Higgins and his colleagues find *L. monocytogenes* a useful model for studying intracellular bacterial pathogenesis, mechanisms of growth and cell to cell spread, and bacterial determinants of the host T-cell response. In work partly supported by a 1999 Armenise Foundation Fellowship Grant, Dr. Higgins developed a versatile system that incorporates a bacterial cytolysin into a vector for delivering proteins of interest to host phagocytes.

Dr. Higgins' team started with an especially harmless strain of *Escherichia coli* , called K-12, and expressed in it cytoplasmic recombinant listeriolysin O (LLO), which efficiently targets proteins to the cytosol of macrophages and dendritic cells, so-called professional phagocytic cells. LLO is a pore-forming cytolysin whose natural biological role is to explode *L. monocytogenes* vacuoles, so that bacteria can escape into the cytosol and replicate. They used this vector to deliver several different proteins to professional phagocytic cells.

For their next series of experiments, the investigators used a novel packaging method called a diaminopimelate (dap) auxotroph to express LLO and *Yersinia pseudotuberculosis* invasin in *E. coli* K-12. The resulting bacterial vector could deliver a protein cargo to the cytosol of non-professional phagocytic cells. Following invasin-mediated entry into mammalian cells, the *E. coli* remained within vacuoles yet underwent lysis because dap auxotrophs are stamped with a kind of biological expiration date. LLO-mediated perforation of the vacuole then allowed release of cargo proteins into the cytosol.

Using T-cell activation assays, Dr. Higgins' team demonstrated that the invasive/autolytic *E. coli*/LLO strain could deliver antigenic protein to the cytosolic major histocompatibility complex (MHC) class I processing pathway, which subsequently presented protein fragments to cytotoxic T-cells. In theory, this system could be used to deliver any antigenic protein for presentation on MHC class I molecules, which could prove very useful for priming protective T-cell responses,

Dr. Higgins said. In the long run, this could be a platform technology for launching new vaccines.

Electron microscopic studies on integrins: activation and ligand binding
Thomas Walz
Department of Cell Biology, Harvard Medical School

Integrins are cell adhesion receptors that mediate the binding and detachment of ligands involved in cell-cell and cell-pathogen interactions. In 2001, Dr. Walz and colleagues determined the crystal structure of the extracellular domain of αVβ3 integrin. Their studies revealed an extraordinary head domain, which they observed in either a "clasped" or "unclasped" position. To determine which position bound ligands more effectively, they used molecular electron microscopy to visualize the structural changes involved in activation and ligand binding.

The crystal structure of αVβ3's extracellular domain revealed a bent conformation, in which the ligand-binding headpiece is folded back onto the tails of the molecule, so that *in situ* the ligand-binding site would be close to the membrane surface. This conformation was thought to be an artifact of X-ray crystallography with no physiological significance, Dr. Walz said. To find out, they exposed the integrin to calcium or manganese, observed what happened, then added ligand to the mix. A combination of electron microscopic studies and ligand-binding assays revealed that the bent conformation, seen in the presence of Ca^{2+}, actually represents the inactive state of the molecule.

In contrast, when integrin αVβ3 was incubated with Mn^{2+}, both the clasped and unclasped forms were activated and their tails fully extended. These observations suggest a "switchblade"-like motion of αVβ3 during the activation process that involves the straightening of the bent receptor. When ligand was added, both assumed the binding position. Dr. Walz's team has now moved on to analyzing changes of shape in a different integrin, α5β1, brought about by ligand binding.

Requirement for a p38 MAP kinase signaling pathway in *Caenorhabditis elegans* innate immunity

Frederick M. Ausubel
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The innate immune system is the body's first line of defense against threats from the outside world. It relies on physical and chemical barriers and the rapid deployment of specialized cells that to right off invaders until the acquired immune system can generate antibodies and T cells. Important insights into mammalian innate immunity have emerged from studies in *Drosophila*, where geneticists have identified key genes that are important not only in flies but in humans as well. More recently, development of experimental host-pathogen models involving bacterial infection of *Caenorhabditis elegans* provides the opportunity to explore evolutionarily conserved pathways of innate immunity in the genetically tractable nematode host. Dr. Ausubel and his colleagues at Massachusetts General Hospital have put this model to especially good use.

In the Ausubel laboratory, a *C. elegans-Pseudomonas aeruginosa* pathogenesis model was used to screen for *C. elegans* mutants with enhanced susceptibility to pathogens. The screen identified two genes required for host defense: *esp-2* and *esp-8*, encoding MAP kinase kinase and MAP kinase kinase kinase components of a p38 MAP kinase signaling cassette in *C. elegans*. When the esp8 gene was knocked out, the nematodes were easily killed by pathogens, Dr. Ausubel said. The defense system was also short-circuited when RNA inhibition was used to temporarily turn off the p38 MAP kinase step in the pathway. In separate experiments using *Salmonella enterica* as the pathogen, programmed cell death in the *C. elegans* gonad (previously shown to protect against *S. enterica*-mediated killing) was found to be downstream of the p38 MAP cascade and dependent on lipopolysaccharide, a component of the Gram-negative bacterial cell wall.

These findings fit into a global picture of innate immunity that has emerged over the past decade. Dr. Ausubel summarized evidence for a MAP kinase signaling pathway in plants, which parallels self-defense mechanisms in animals as simple as *C. elegans* and as complex as humans. Recent findings in *C. elegans* validate the use of this organism as a model for studying innate immunity. More importantly, these experiments add weight to the idea that the MAP kinase cascade is "the most ancient and conserved component in innate immunity," Dr. Ausubel said.