Current Topics

FDA, Producers Moving toward Mammalian Cell-Based Flu Vaccines

Manufacturers and officials of the Food and Drug Administration (FDA) are planning a major shift in how subunit influenza vaccines are produced—discarding chicken eggs in favor of cultured mammalian cells (Microbe, January 2006, p. 4). Several factors are adding momentum to this pending shift, including concerns over pandemic flu, a pledge by President Bush for new resources to support research on producing vaccines in cell cultures, and a general move to eliminate bottlenecks embedded in the traditional approach to making flu vaccines using eggs. Despite this momentum, however, vaccine producers will need to surmount safety concerns before FDA officials approve vaccines made using this approach (ASM News, May 2004, p. 210).

At least two flu vaccine manufacturers, Chiron of Emeryville, Calif., and Solvay Pharmaceuticals of Marietta, Ga., and Brussels, Belgium, are producing candidate subunit-type influenza vaccines through use of Madin-Darby canine kidney (MDCK) cells. Such cells were first extracted in 1958 from a healthy cocker spaniel and subsequently “immortalized” through growth in culture. Such cells prove to be “highly permissive” for growing the influenza virus, says Rina Rappuoli of Chiron, who spoke last November during a meeting of the FDA advisory committee, whose members reviewed safety concerns surrounding this use of MDCK cells.

Influenza viruses grow to high yields in MDCK cells, providing a “favorable ratio of virus to impurities,” thus making this approach to making vaccines “economically feasible,” adds Jeroen Medema of Solvay, who also spoke during the November advisory session. His company recently built a facility in the Netherlands, in which MDCK cells are being used to produce flu viruses for an experimental vaccine. In the Solvay process, the MDCK cells are grown on microcarriers within bioreactors. European regulatory officials granted the company a license for producing subunit flu vaccines in this fashion, and more than 1,000 Europeans have received a candidate flu vaccine in clinical trials, he says. Immune responses among recipients are “comparable” to those seen with conventional vaccine, and there were “no unexpected safety findings.”

Half a century ago, the U.S. Armed Forces Epidemiological Board declared that “only normal” cells would be used for making vaccines. Since then, many different types of cells came into use for making vaccines, and MDCK cells are considered the “logical next step in this progression,” says Philip Krause of the FDA Office of Vaccines Research and Review. Yet, despite advantages in using MDCK cells, there are concerns about them being tumorigenic and presenting other risks, including from adventitious agents, residual DNA, and virus-cell interactions. Moreover, they also present a “perception” of increased risk. With such concerns in mind and after meetings with outside scientists, FDA officials devised a “defined risks approach” for evaluating the safety of flu vaccines produced in MDCK cells, he says, noting that this approach provides a “quantitative conceptual framework for estimating upper bounds on potential risks.”

One unknown is whether MDCK cells are not only tumorigenic, but also oncogenic—meaning they might contain agents that “transform the cells of the injected species into neoplastic cells that grow into tumors,” says Krause’s colleague Andrew Lewis. If the new vaccines were to cause such unexpected types of tumors in animal tests, he suggests, it “would make our hair stand on end.”

The Chiron and Solvay approaches, although both based on MDCK cells, differ in ways that could bear on safety. Unlike the Solvay process, which uses polarized MDCK cells contained in microcarriers, the MDCK cells being used by Chiron are adapted to grow in suspension. Although both types of cells can induce tumors when administered to immunosuppressed mice, the use by Chiron of MDCK cells that grow in suspension adds further uncertainty about the potential for tumorigenicity and oncogenicity, according to several members of the FDA advisory committee.

Still, these concerns fall in the low-probability range. MDCK cells are well established, have been used routinely in research labs for several decades, and are “remarkable” for their safety features, according to Chiron’s Rappuoli. Moreover, such cells can be more thoroughly characterized than can “primary cells,” namely those freshly extracted from animals. And MDCK cells can be grown on chemically defined media, eliminating a need for serum supplements, whose
use risks introducing prions and other adventitious agents.

Both the Chiron and Solvay post-MDCK vaccine-manufacturing procedures entail extensive purification and inactivation steps to reduce the risk that any intact MDCK cells or cell-derived neoplastic agents could be found in the final vaccine product. Solvay’s Medema estimates a greater than $10^{21}$ clearance factor for removing MDCK cells from finished vaccine, while Rappuoli’s cell-removal estimates extend higher.

Although anxiety about pandemic preparedness is a major driver for shifting to this new means of producing flu vaccines, several experts on the FDA advisory committee point out that a whole virus-based vaccine is more likely than subunit vaccines to protect against an emergent flu virus during a pandemic. However, the extensive purification steps devised for these two MDCK-based production schemes are not suited for making whole-virus vaccines. Nor do the two companies plan to revise those schemes. “We have no intention to make a whole-virus vaccine,” Rappuoli says. “With an adjuvant, we can get a good response with a subunit vaccine. The egg vaccine [for flu] is 1950s technology.”

Assuming that safety concerns can be addressed, Chiron and Solvay appear poised to dispense generally with that production technology in favor of the MDCK-based process for producing flu vaccines. Indeed, using MDCK cells is more versatile for scaling up or shifting to cover emerging flu strains, cuts production time by about six months, and sidesteps the difficulties of adapting sometimes lethal (for eggs) flu strains to grow in eggs and of procuring chickens, which can become scarce when avian flu is rampant.

**Carbon Dioxide Orchestrates Growth, Virulence of Some Fungi**

Carbon dioxide helps to regulate respiration in many species, drives photosynthesis in plants, and serves as an attractant for insects such as mosquitoes. On the microbial level, this gas, which readily hydrates to bicarbonate, governs morphologic changes and other behaviors that affect virulence and survival of two fungal pathogens, Cryptococcus neoformans and Candida albicans. Members of separate research teams, one in the United States and the other in the United Kingdom, report their findings in the November 2005 issue of *Current Biology* (15:2013–2020, 2021–2026).

Both these pathogens and other microorganisms face very different levels of carbon dioxide, depending on where they are situated. In air, carbon dioxide gas levels amount to 0.036% compared to 5–6% of the dissolved gas that the fungal cells encounter inside human or other animal hosts. *C. neoformans* cells somehow sense those carbon dioxide levels, which can affect metabolism, growth rates, and sexual reproduction, according to microbiologist Joe Heitman at Duke University Medical Center in Durham, N.C.

The enzyme carbonic anhydrase (CA), which speeds the conversion of carbon dioxide gas to soluble bicarbonate, plays a key role in promoting the growth of *C. neoformans*. Mutants lacking CA and exposed only to atmospheric carbon dioxide grow poorly because they fail to make adequate bicarbonate, which is essential for fatty acid biosynthesis, Heitman says. However, when the same cells are exposed to high levels of carbon dioxide within mammalian hosts, where the conversion to bicarbonate occurs spontaneously, they thrive.

Carbon dioxide affects not only growth rates, but also several *C. neoformans* behaviors, according to Heitman. For example, the cells mate freely in ambient air where carbon dioxide levels are low. Once exposed to elevated levels of this gas, the cells stop sexual mating and no longer form spores, a process controlled by CA. Humans become infected by in-

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haling *C. neoformans* spores, so “it’s important to understand its natural life cycle and the environmental signals that control it,” he says.

About 20 years ago, researchers learned that carbon dioxide induces *C. neoformans* to form a polysaccharide capsule when it infects hosts. Now Fritz Mühlischlegel of the University of Kent, United Kingdom, and his collaborators confirm that this process depends on carbon dioxide activating adenylyl cyclase. In *C. albicans*, the same team shows that high levels of carbon dioxide trigger a reversible transition from the benign yeast-like form to the virulent filamentous form, and that adenylyl cyclase, which generates cyclic AMP, acts as a chemosensor.

When *C. albicans* cells infect skin, a low-carbon-dioxide niche, the cells depend on CA to generate bicarbonate, according to Mühlischlegel. “In superficial infections, the partial pressure of carbon dioxide is too low to yield bicarbonate via spontaneous chemical reactions,” he points out. In contrast, the rich supply of carbon dioxide available during systemic infections allows fungal cells to dispense with CA.

Identifying carbon dioxide sensing along with the involvement of adenylyl cyclase and CA in fungal pathogenesis suggests new targets for therapeutics. “There are several possibilities for interfering with carbon dioxide sensing, and we are testing a number of compounds,” Mühlischlegel says. Heitman calls carbon dioxide an “important signal,” perhaps a general phenomenon, noting that it also controls some stages of virulence in other pathogens, such as *Bacillus anthracis* and *Coccidioides immitis*.

Heitman and Mühlischlegel, who learned of one another’s investigations when they met during a conference, suspect that carbon dioxide bypasses the usual cell surface receptors to interact directly with adenylyl cyclases. They propose that it diffuses through cell membranes and is converted to soluble bicarbonate, which then binds directly to adenylyl cyclase. “It’s hard to conceptualize what senses gases,” says Heitman, who predicts an upcoming “renaissance in the way that we think about how soluble gases are sensed and transported.”

The evidence is mounting that carbon dioxide and other gases that are readily hydrated, such as ammonia, play broad biological roles in sensing and development, according to microbiologist Sydney Kustu at the University of California, Berkeley. Her work indicates that Rhesus proteins, which confer Rh blood types, serve as carbon dioxide channels through cell membranes. “Previously, their function had been unknown for 65 years,” she says.

**Carol Potera**
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### Salmonellae Alter Host Responses through Direct T Cell Contacts

*Salmonella* bacteria can disable mammalian T cells, shutting down these key cells of the immune system with a factor that is released only after the bacteria and T cells directly contact one another, according to microbiologist Michael Starnbach of Harvard Medical School in Boston, Mass., postdoctoral fellow Ando van der Velzen, and their collaborators. This capacity to shut off or reduce an infected host’s immune response may explain how some individuals become long-term carriers of these bacteria. The Harvard researchers reported their findings in the December 6, 2005, issue of the *Proceedings of the National Academy of Sciences* (102:17769–17774).

When *Salmonella* cells are engineered to express an ovalbumin-derived peptide as a marker antigen, they fail to stimulate an immune response to this peptide, according to Starnbach. Because earlier studies showed that *Salmonella* can kill dendritic cells, he suspected that the engineered *Salmonella* might be acting through dendritic cells to shut down the expected T cell response.

To test this, the researchers engineered *Salmonella* that lacked the genes necessary for killing dendritic cells, as well as dendritic cells that were resistant to the death signals delivered by *Salmonella*, and tested each in turn to see whether T cell responses might be restored. “It wasn’t leading...
us to find the direct contact-dependent effect we report in this paper,” says Starnbach. Thus, in addition to the disabling effects of salmonella on dendritic cells, the bacteria also can act directly on the T cells, according to Starnbach and his collaborators. Indeed, when Salmonella and host T cells are separated by a filter that prevents them from touching, “we didn’t get inhibition,” he says. However, once Salmonella contact the T cells, a factor is secreted that prevents T cells from proliferating. “We are attempting to purify the factor, and to identify it biochemically, as well as to identify the gene(s) encoding the factor,” he says. Those T cells that directly contact bacterial cells are most affected, Starnbach says. There are “probably other T cells at the site of infection that don’t come into contact, and many others elsewhere in the body” that can contribute to a successful defense against Salmonella infections, he says.

“Starnbach and colleagues provide an interesting new twist to this story, clearly demonstrating that Salmonella can directly interact with T cells to prevent their proliferative response to T cell receptor ligation,” says John Harty of the University of Iowa in Iowa City, who was not involved in the research. Importantly, this response occurred even in T cell stimulation assays without dendritic cells and thus, these studies identify a novel immunosuppressive pathway used by Salmonella to limit host responses to infection. “Of further interest, this pathway did not depend on the classically defined virulence mechanisms of Salmonella,” he adds.

Salmonella infections in the United States each year cause an estimated 1.4 million cases of food poisoning. In some cases, these infections persist indefinitely, as in the infamous case of “Typhoid Mary” Mallon, a domestic cook in New York around the turn of the last century, who infected more than 30 people before she was quarantined, and whose gallbladder was found to be teeming with the pathogens.

David Holzman
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Deep Ice Harbors Plentiful, Culturable, but Ultrasmall Microbes. . .

Most living microbes that were found in an ice core sample from a 3,043-m-deep Greenland glacier are less than 1 μm in size, smaller than typical bacteria that range from 1–10 μm, according to Vanya I. Miteva and Jean E. Brenchley of Pennsylvania State University, University Park. “We detected numerous ultrasmall cells in the melted ice filtrates, and successfully cultivated small-celled isolates at 5°C,” Miteva says, alluding to a surprisingly wide variety of viable microbes that were found in ice layers, which were deposited at least 120,000 years ago (ASM News, September 2004, p. 392). Further details appear in their report in the December 2005 Applied and Environmental Microbiology (71:7806–7818).

Among the hundreds of microorganisms from the ice core, “at least 14 isolates” appear to be “previously unknown organisms, [adding] substantially to the very short list of model ultrasmall microorganisms,” Miteva says. Within that submicron range, the cells come in “many different shapes and sizes” when viewed by electron microscopy, she adds. “These results extend our knowledge about the ability of cells to withstand extreme conditions that might exist elsewhere in our solar system. Glacial ice is considered to be a good analogue for extraterrestrial life.”

These findings add to other “evidence that single-celled organisms can adapt to a combination of incredibly harsh conditions,” says Buford Price of the University of California, Berkeley, whose own study found methanogenic microorganisms in similar ice cores (see next story). “It is not a great leap to conclude that if life arose on Mars or Europa when conditions were less hostile, it may still survive today in favored locations.

“I think it is important that the smallest cells they were able to see were about 0.2 μm by 0.2 μm by 0.2 μm,” Price continues. “This is safely

CDC Proposes New Rules for Quarantines, Other Safeguards against Disease Outbreaks

Officials at the Centers for Disease Control and Prevention (CDC) in Atlanta, Ga., last November proposed new safeguards for dealing with infectious diseases, particularly for those affecting international travelers arriving in the United States. The proposals will expand reporting of ill passengers who are on interstate flights and those arriving from foreign countries, require that ships and flights from foreign countries (and some interstate flights) submit passenger and crew lists electronically to CDC upon request, and also spell out due-process procedures for individuals who are subject to quarantine. These proposed rules address procedures for keeping individuals who might be infected with any of nine specific diseases from entering the country, including pandemic influenza, cholera, diphtheria, infectious tuberculosis, plague, smallpox, yellow fever, viral hemorrhagic fevers, and severe acute respiratory syndrome, or SARS.

Centers for Disease Control and Prevention
above the lowest size that theoretical biologists claim is necessary to house all the constituents necessary for self-replicating life.”

The findings also provide fresh evidence that ultra-small microbes exist—a matter of continuing controversy. Just why so many of the microbes in the core samples are so small is yet another matter for debate. For instance, “Many cells that are small in natural samples have become small by starving, and when they are provided nutrients, they increase in size,” says Rick Cavicchioli of the University of New South Wales, Sydney, Australia.

But Cavicchioli notes that this wasn’t the case in this study. Says Miteva, “More than 85% of our ultrasmall-celled isolates maintained their small sizes after being cultured repeatedly in nutrient-rich media.” Moreover, they appear to be “related to other ultrasmall microorganisms found in other environments.” Another possibility for the unusually small sizes, according to Brian Lanoil of the University of California, Riverside, is that smallness provides a means for “surviving in low-nutrient and/or low-temperature environments.”

Never mind size, the remarkable finding is that so many different kinds of unusual microbes can be grown in culture after being extracted from deeply drilled glacier ice, argues Julia Foght of the University of Alberta in Edmonton, Alberta, Canada. “While molecular methods may still paint the most colorful pictures of biodiversity, it is cultivation that facilitates full characterization of the microbes by revealing metabolic potential, microbe-microbe interactions, gene expression profiles, et cetera,” she says. “The multifaceted approach to chasing down these otherwise elusive microbes is to be applauded,” Foght continues. Applying it more broadly could help to overcome the “defeatist attitude” among some investigators who figure that many microorganisms simply cannot and may never be grown in culture.

David Holzman

...and Marsh-Gas Microbes from Glacial Ice Seem Consistent with Martian Life

Samples from 3,053-m-deep ice cores from glacial ice that overlays Greenland contain methanogenic bacteria, whose earthly presence carries interplanetary implications, according to Buford Price and his collaborators from the University of California, Berkeley (UCB). They speculate that bacteria, if found in similarly icy sites on Mars, could be the source of that planet’s atmospheric methane, which also is called marsh gas.

Antibiotics Implicated in Emergence of Highly Virulent C. difficile Strain

Fluoroquinolone use may be driving the emergence of a particularly virulent strain of Clostridium difficile that causes severe diarrhea and is turning up regionally in parts of the United States and Canada, according to two studies reported in the December 8, 2005, issue of the New England Journal of Medicine. This strain is resistant to fluoroquinolone, produces an atypical binary toxin, and contains a characteristic 18-base-pair deletion in the tcdC gene, which is thought to encode a regulator of toxin genes in this microbe, according to L. Clifford McDonald of the Centers for Disease Control and Prevention (CDC) in Atlanta, Ga., and his collaborators. Prevention efforts, especially scrupulous hygiene in hospitals, and “antibiotic stewardship” will be “particularly important” for managing the epidemic that this strain threatens, according to John Bartlett and Trish Perl of Johns Hopkins University in Baltimore, Md., whose editorial accompanies the two reports.
Moveable ORF Provides Feast for Combing Yeast Genome, Expanding Glycome

The genome of *Saccharomyces cerevisiae* consists of more than 12 million DNA base pairs, encoding more than 6,600 open reading frames (ORFs). Michael Snyder of Yale University in New Haven, Conn., Elizabeth Grayhack of the University of Rochester Medical Center in Rochester, N.Y., and their colleagues recently constructed a special version of a yeast genomic library, one that contains more than 5,000 of those ORFs with a movable, C-terminally tagged expression vector—something that they are calling a “moveable ORF,” or MORF. With this MORF, they built a chip containing 5,573 purified proteins from which they identified 109 new N-linked glycoproteins, nearly doubling the contents of the yeast glycome. Apparently, having that C-terminal tag allows those expressed proteins to enter the yeast secretory pathway where they undergo glycosylation and are available for other posttranslational modifications, the researchers note in the (early) December issue of *Genes and Development*. Moreover, identifying more than 100 new confirmed N-linked glycoproteins and 345 candidate glycoproteins “in a single pass illustrates the power of screening an entire proteome at once.”

As part of Greenland Ice Sheet Project 2, the UCB researchers analyzed samples from deep cores extracted from glacial ice overlaying bedrock. They were provided those samples by researchers from the National Ice Core Laboratory in Denver, Colo., who found high concentrations of methane at depths of 2,954, 3,018, and 3,036 m.

Price and colleagues analyzed a series of samples from depths ranging from 500 to 3,000 m for microbes, looking specifically for methanogens on the basis of their distinctive F$_{420}$-coenzyme autofluorescent signal. That signal was compared to those from authentic samples of *Methanococcus jannaschii*, amid painstaking efforts to ensure that the core samples were not contaminated with ordinary microbes, according to their report in the December 20, 2005 issue of the *Proceedings of the National Academy of Sciences*.

The methanogens that they found were embedded only in methane-rich zones, particularly those from depths of 2,954 and 3,036 m. Meanwhile, samples from nearby depths, either 1 m above or below those “hot spots,” contain few bacteria and little methane, according to Price and his collaborators. Although oxygen is present at such depths, it tends to be “locked up” in cage-like structures known as clathrates. Frozen in place and unable to divide appreciably, the methanogenic microbes from the core layer samples survived in these narrow anaerobic niches for roughly 200,000 years, Price reckons.

Finding metabolically active microbes deep within glacial ice is not so surprising, according to Karsten Pedersen, head of the Deep Biosphere Laboratory at Göteborg University in Sweden. “We find microbes 1,000 m under ground,” he says. “It is cold, but that only slows biological reactions.” Earthly environments that appear not to contain active microbes include those that are warmer than about 115°C and those that are very, very dry, he adds.

Price and his colleagues point to recent measurements of methane levels in the Martian atmosphere and say that biological activity may be needed to sustain those levels. “We know from our study of the rate of methane generation as a function of temperature that the [Greenland] methane is generated at the right rate [to sustain the Martian atmosphere] if the methanogens are at a temperature around 0 to 10°C,” he says. “From the work of others, we know that the Mars subsurface temperature is 0 to 10°C at a depth of 150 m to a couple of kilometers.” Thus, in light of the Greenland glacial core sample studies, the Martian marsh gas seems consistent with methanogenic microbial life on Mars— but is still short of proof.

**Brian Hoyle**

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Reovirus Targets Tumors, Looks Promising in Early Clinical Trials

Reovirus selectively destroys mammalian cells containing activated Ras signaling pathways. Because some two-thirds of human tumors carry such activated pathways, reovirus holds potential for treating cancer, according to Patrick Lee, formerly at the University of Calgary in Alberta, and now at Dalhousie University in Halifax, Nova Scotia, Canada, and his collaborators.

“We were looking at viral receptors, not for a virus that targets cancer cells,” Lee says, referring to a project that began about a decade ago. However, he and his collaborators then learned that reovirus hijacks the activated Ras signaling pathway in tumor cells and begins producing its own proteins at the expense of those needed by cancer cells, undercutting their chances of survival. The Ras
pathway is considered an important regulator of growth, and is distributed extensively, perhaps universally, among eukaryotic cells. A protein in such cells, called RNA-activated protein kinase (PKR), blocks reovirus from replicating and harming them. However, tumor cells with activated Ras pathways lack PKR activity, allowing reovirus to replicate freely. Nearly two-thirds of human tumors are thought to carry activated Ras pathways.

In 1998, Lee and colleagues James Strong and Matthew Coffey co-founded Oncolytics Biotech, Inc., in Calgary to develop reovirus as an anticancer agent. “Reovirus usurps all the tumor cell translation machinery,” says Coffey, chief scientific officer at Oncolytics. After reovirus-infected cancer cells begin generating reoviruses, many other functions required for cell survival cease, then apoptotic or necrotic events occur, and they “lyse like overfilled balloons,” he says.

“Reovirus is very stable and robust,” Coffey says, noting that the Oncolytics experimental therapeutic, called Reolysin, consists of unattenuated reovirus suspended in saline. It appears to be safe when administered to patients, based on clinical trials in Canada. Doses as high as 1 billion infectious particles are injected directly into solid tumors. About 60% of patients in this trial (11 of 18) showed some response to the viral therapy, and tumor regression ranged from 32 to 100% for a variety of tumors in end-stage patients who failed to improve with other therapies. Moreover, noninjected, remote tumors also regressed. During the next round of clinical trials, physicians will evaluate how patients respond when reovirus is combined with radiotherapy; they also will evaluate whether it is effective for treating metastatic tumors when administered systemically.

Reolysin alone or radiation treatment alone slows the growth of B16 melanoma implanted into mice, whereas Reolysin plus radiation blocks cancer cell growth for up to 35 days. Investigators described this synergistic effect during a conference on cancer therapeutics held last November 2005 in Philadelphia, Pa. In addition, last July patients in the United Kingdom began enrolling for a clinical trial evaluating combined Reolysin-radiation treatments.

Lee continues to study molecular mechanisms behind reovirus infection. In experiments that compare reovirus actions in normal and cancer cells, “transcripts are made equally well, but translation efficiency is much higher in cancer cells,” Lee says. Another avenue of research follows upon earlier animal studies that showed that reovirus works best when immune system activity is depressed. Lee is addressing the molecular mechanisms behind this process. These basic biology experiments will “improve our understanding of cancer biology and reovirus therapy,” he says. For instance, reovirus in combination with immune-suppressing drugs may benefit some patients with particular types of tumors.

The double-stranded RNA reovirus, whose name is an abbreviation for “respiratory enteric orphan virus,” was first isolated in 1951 from human feces. The virus is found in sewage and water supplies; when it infects humans, typically it causes little more than mild runny noses. Albert Sabin named the virus “reo” because it lives in the body’s respiratory and enteric tracts, and he considered it an “orphan” because it does not cause any obvious disease, despite its similarities to poliovirus.

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