PLANT-DERIVED BIOLOGICS MEETING

April 5, 2000, 8:00 a.m.

Scheman Building Auditorium

Ames, Iowa

Reported by: SueAnn Graham, CSR, RPR

I N D E X

P R O C E E D I N G S

JIM ROTH: Good morning. We'd like to get started. We have a very busy program, so we will try and stay on time and get started promptly and get the breaks promptly on time.

I am Jim Roth. I am with the Institute for International Cooperation in Animal Biologics and with the College of Veterinary Medicine here at Iowa State University.

IICAB is hosting this event, so if you have logistical problems or need things, you can contact me or Jane or Dawn at the desk.

I would like to introduce Dean Richard Ross from the ISU College of Veterinary Medicine to give a welcome. Dr. Ross, as I said, is the dean of the College of Veterinary Medicine. He's also serving as interim dean of the College Agriculture while we're doing a search for that college, so he's wearing two hats and being quite busy. We're really glad he's able to come this morning.

Dr. Ross is a veterinary and a mycoplasmologist. He's past president of the International Association of Mycoplasmologists, very active and played a key role in developing mycoplasma vaccines for swine. So he's also a content expert in much of what's going to be going on at this meeting.

Dr. Ross is past president of the American Association of Veterinary Medical Colleges. He also serves on the Secretary of Agriculture's National Agricultural Research Extension Education and Economics Advisory Board.

So Dean Ross.

RICHARD ROSS: Thank you very much, Jim. It's a pleasure for me to represent the College of Veterinary Medicine and Agriculture in welcoming you to Iowa State's campus for this very exciting seminar on plant-derived biologics.

Iowa State University and the State of Iowa are moving aggressively to establish leadership in the plant sciences area. Those of you in the Ames area probably are pretty familiar with that, but I wanted to give you just a glimpse of what's going on and broadening the plant sciences. Frankly, I think it's fundamental to the applied topic that you're talking about today.

With significant private support, almost 100 million this last year, in just the last few months, very strong

support from the State of Iowa, the university has established a world-class plant sciences institute with a series of centers.

It brings together the best minds in the world of collaborative programs in academia, government, private sector, and research and technology transfer, optimizing all the advances that are taking place in the plant sciences.

Developments in genetics, genomics, plant transformation, and elements of information sciences, mathematics, statistics, now BIOINFOMATICS, are drawing increased attention to the plant sciences and universities and government research agencies and life science companies.

State University has been recognized for many years for its strengths in agronomy, seed science, molecular biology, biochemistry, chemical engineering, animal science, veterinary microbiology, and veterinary virology.

So what we see, I believe, is a merging, a fusing, of a lot of these traditional disciplines into a broad interdisciplinary thrust in plant sciences and then utilizing plants to develop new products for use for humans across the world.

An interdisciplinary thrust that has been underway that Dr. Roth alluded to, a cooperative venture between Iowa State University, the Center for Veterinary Biologics, National Veterinary Services Laboratory, National Animal Disease Center here in Ames is the Institute for International Cooperation in Animal Biologics. So I think the IICAB is well positioned to capitalize on some of the plant sciences initiatives that are taking place here at Iowa State.

The objectives of IICAB, just to remind you, those of you who weren't here a couple of days ago when Dr. Roth gave an overview of activities in IICAB, are to facilitate international exchange of information of importance to veterinary biologics regulatory authorities and producers and users, to serve as an international resource center for veterinary biologics that assists in training, technology, development, and supply of references and reagents, to facilitate the establishment of international standards in animal biologics and harmonization of standards, regulations, and products, and fourthly, to assist countries in obtaining veterinary biologics for specific needs in those countries.

The organizers of this conference have worked diligently to develop an excellent program, and they cover

an array of the application of plant science technologies to use in the development of animal biologics.

There are some other very important developments coming out of these activities, and I just want to remind you of them. There are developments of exciting new plant varieties, for example, rice and Vitamin A and iron that can help to reduce or mitigate the roughly 500,000 children that end up with blindness every year, absolutely phenomenal development with great potential to improve the health of children. Many, many other examples of plant varieties of that nature.

Another improvement that comes from these plant sciences ventures, improve health and growth performance of livestock, some potentialfor biomediation, new biomass products, new synthetics for manufacturing medicine and clothing, pharmaceuticals, and potentially even mechanisms to address the neoplastic and genetic disease. And finally, of course, the topic for today, vaccines for animals and a generation of antibiotics in plants as well as for use in animals.

The workshop, you have the -- I guess it's six breakout sessions that are listed in the program, and they go through an array beginning with the expression systems and ending up with regulatory considerations. I'm excited about the program. I'll try to attend, and I look forward to the information that will be transferred and discussed during the conference.

Thank you, Dr. Roth, and welcome.

JIM ROTH: Thank you, Dean Ross. Now I would like to introduce Dr. Louise Henderson. Dr. Henderson is a molecular biologist. She received her Ph.D. from Iowa State University. She's been with the USDA since 1988.

Officially, she's with the USDA-APHIS VS/CVB/LPD, and I need to tell you what that stands for. APHIS, of course, is the Animal Plant Health Inspection Service. Veterinary Services is a division under that. And then she's with the Center for Veterinary Biologics, which is located mostly here in Ames, Iowa, and that's the grou that regulates all of the veterinary vaccines in the U.S. and all vaccines for export for animals from the U.S.

Within CVB Dr. Henderson is with LPD, which is Licensing and Policy Development. That's the group that issues the licenses for any veterinary vaccine and develops the policies, including the policies on transgenic plants and plant viruses to be used as vaccines.

And, Dr. Henderson is chief of the biotechnology and diagnostics group within LPD, so she'll be a key person in developing the policies on plant-derived biologics.

Dr. Henderson.

LOUISE HENDERSON: Thank you, Dr. Roth. Before I welcome you, I have a housekeeping chore, and that is that there is a blue Buick with South Dakota plates 61D 035 that has its lights on in the parking lot. You may want to check into that. Otherwise we'll be checking with Dr. Roth to see if he has jumper cables --

JIM ROTH: I do.

LOUISE HENDERSON: -- this afternoon. It is a privilege to welcome you all on behalf of the USDA campus. For those of you who don't know it, this program has grown out of a seminar program from the USDA campus, and that campus includes the National Veterinary Services Labs, which is a leading research organization in veterinary disease, diagnosis, and prevention and cure.

The National Veterinary Service Laboratories, which is the national reference laboratory responsible for many activities that are necessary for control, detection, and eradication of foreign animal diseases and on program diseases within the U.S., and they play a key role in making sure that foreign animal diseases do not establish themselves in this country which would have a huge impact on our agricultural situation.

And then finally, the Center for Veterinary Biologics, which Dr. Roth explained that we do regulate and oversee all of the licenses for all veterinary biologics.

We're really excited to have as co-coordinators of this program APHIS Plant Protection and Quarantine. Those of you who are in the plant field realize that they play a key role in regulation of growing all of these types of plants in the environment and will continue to play a key role in regulation of these products, whether they're intended for biologics or for other uses.

And also, the Food and Drug Administration Center for Biologics Evaluation and Research, CBER, which it's charged with doing much the same as we do but for human vaccines and biologics.

We think this is a unique opportunity for our agencies to work together and an opportunity to do more with less, if you will, and not have bifurcation of government services. We think this is a very unique time in which we can develop policies together.

The purpose of the meeting is to open an inter-agency, industry, and public dialogue on plant-derived vaccines, therapeutics, and diagnostics. And those of us charged with regulating various aspects of this encourage you to participate fully and actively.

This dialogue can be more meaningful with your participation. This is an opportunity for you to tell us what you're thinking as well as for you to share information with us.

We intend to develop some policies and coordinate that policy development between the agencies, and we hope that you will have lots to tell us about what you think needs to be done and tell us your perspective on these policies because we want to make sure that our policies have a strong basis in science and also facilitate the use of this technology while still protecting consumers and the environment. So thank you very much for coming.

And with that I'll turn it back to Dr. Roth.

JIM ROTH: Thank you, Dr. Henderson. Next I would like to introduce Dr. Katy Stein of the FDA CBER. Dr. Stein is an immunologist. She did her Ph.D. at Albert Einstein College of Medicine, post-doc at Harvard and NIH. She's been with CBER since 1980. And now I need to tell you what CBER is. CBER is C-B-E-R, is the Center for Biologics Evaluation and Research within the Food and Drug Administration. And CBER has responsibility for regulating human vaccines.

So this is an excellent opportunity, this meeting, to interact with the people that will be developing policies and regulating both animal vaccines and human vaccines.

Dr. Stein is the Director of the Division of Monoclonal Antibodies within the Office of Therapeutics Research and Review of CBER.

Dr. Stein.

KATHRYN STEIN: Good morning. On behalf of the FDA and all of my colleagues here from the Center for Biologics Evaluation and Research, the Center for Drug Evaluation and Research, the Center for Veterinary Medicine, and the Center for Food Safety and Applied Nutrition, I want to welcome all of you this morning.

I think as Louise mentioned, there are many common issues that will determine policy for both the FDA and the USDA as we go forward and regulate plant-derived biologics.

We're very excited about this meeting. We do hope to have input and questions from all of you.

There are many FDA colleagues here, and you should feel free to contact us to discuss your issues and give us your insights into plant-derived biologics.

It is now my great pleasure to introduce Dr. Jose Luis DiFabio from the Pan American Health Organization who I think will set the stage for the great need for inexpensive plant-derived easily deliverable vaccines for the developing world, and I think we'll put all of these issues in perspective.

Dr. DiFabio is a chemist, having derived his training at the University of British Columbia in Vancouver. He worked for a number of years at the Bureau of Biologics in Canada and is currently the coordinator for the vaccine technology access program within the Division of Vaccines and Immunization at PAHO.

Dr. DiFabio.

JOSE LUIS DiFABIO: Good morning to everybody. I would like to thank the organizers for inviting me to this challenge, of being in front of an expert audience and trying to talk a little bit about public health perspective.

Anyway, there will be for me a challenge to be talking in front of you, and the success or not of my presentation, then you can always blame them on the organizers that invitedme.

What I will be concentrating, as Katy mentioned, mainly on the vaccine issues as this is the area we work in in our organization, the Pan American Health Organization

As you know, vaccines are among the most affordable and effective health interventions available today. That's what we, as a health organization are saying. We have major examples with smallpox. A vaccine has been developed, produced, and by its widespread use has eliminated that disease.

Similarly, we have examples with the polio vaccine in the western hemisphere. Through its use, we haven't had polio in the Americas for several years now. At the moment, the organization is trying to achieve a similar goal with measles.

Vaccines are very important. Even the financial institutions, which look at things from a different perspective, like cost-effectiveness, cost-benefits, they also consider them as one of the most cost-effective health

interventions. Vaccination goes across socio-economic classes. The moment you vaccinate a rich or a poor child, both will be protected.

Although vaccines have been discovered and known for a long time, the first vaccine is over 200 years old, the smallpox vaccine, major developments have occurred only recently. Now we have about 35 vaccines available.

If we have a picture about the disease and mortality all over the world, we can see a few deaths occurring, most of them occurring in medium and low-income countries. We can see that the burden is really hitting mostly the poor. The major diseases responsible for death are respiratory and diarrheal, and HIV has a big impact in Africa. There are over one and a half million deaths occurring with childhood diseases for which vaccines are available like measles, polio, diphtheria, pertussis.

A large number of deaths are occurring due to tuberculosis and malaria, and no vaccines are available. Research has been going on for the last 20, 30 years we are still in the process of obtaining them.

If we look, at morbidity, the numbers are really alarming, four billion cases of diarrhea diseases a year. It's almost 400 million respiratory diseases. We have other diseases like Hepatitis B, Hepatitis C that really will be affecting at older ages such as chronic diseases or cancer.

So what's the present situation? There are over 300 infectious agents recognized, and from the fact of being infectious, even we can expect that a vaccine should eventually be available to tackle those diseases.

There are more infectious agents being associated with other diseases like cancers, chronic conditions that we were not aware of that could be attacked with, vaccines like papilloma virus, for cervical cancer, and other pathogens associated with gastric ulcers or cancers (H. pylori).

We have only 35 vaccines available for infectious diseases. There is quite a large amount of money being spent on R&D.

When we look at the future agenda for new vaccines, this shows the list of what is available now. A list of what will become available soon, but as you can see we already have some being licensed and changing into the available list, like S pneumoniae. The vaccine is now available. There is also a meningococcal C vaccine available. So the picture is changing rapidly. They are moving quickly into the licensed group.

And for the future we are going to have vaccines against these other diseases. But, while vaccines that are changing columns are those of importance for the high-income countries, for those vaccines of importance for the developing world, not enough work is being conducted.

What about vaccinations? The present situation. So we know that vaccination can control some major diseases and we have examples. Over 90 percent of the global birth cohort has access or can be reached for vaccination, but unfortunately, we have only 80 percent average coverage of the six basic vaccines: BCG, DTP, polio, measles. And for some parts of the world like Africa, the coverage is well below under 50 percent.

We have problems with vaccine delivery. We need cold chain. The vaccines that are available need refrigeration, need care because of their thermal stability.

Immunization safety issues, because most of the vaccines are injectable, syringes are needed. Syringe use means they have to be disposed safely, as reusing syringes has all the safety problems we know.

Multiple injections. Most of them are injectable, and so when you have access to a child, you apply some of the vaccines, and then you might not see that child again because of the discomfort that that generated. So multiple injection is a problem in the developing world for the second access to the child.

Transportation. Roads are not there in many places, so we have difficulties in accessing communities, so we have difficulties in accessing children. And the other important component is trained human resources. In the developing world the more you get trained, the more you are worth. So by training people in the immunization process, when they get the appropriate training, then they may find some different job, so you have to retrain new people again.

Even with all these problems, we are saving around three million lives every year, but unfortunately, there are still four million deaths that could be averted with the existing vaccines.

As an example. If you compare the immunization problems of developing countries, and the developed world, we can see that with time, although we all started with the six basic vaccines in the immunization schedule, very quickly the developed world started introducing newer vaccines, and now there are around 13 vaccines, as part of the immunization schedule of a child, while in some parts of the world they are only seven.

In Latin America we are a little bit more fortunate, the gap is not so large, If we consider prices, for the Pan American Health Organization Rotatory Fund, or the WHO or UNICEF, the prices for the six basic vaccines costs less than $1 to fully vaccinate a child.

When we are rubella, mumps, we are close to $1.50. When we're Hepatitis B, we are close to $3. When we add the H. influenza, now we are talking of $12. If we consider the Varicella vaccine, the price of this vaccine alone is around $15. The pneumococcal vaccine that was recently licensed is $60 a dose, so you need around $200 to immunize one child. And the meningococcal conjugated vaccine, for Group C is around $30 a dose, and you need three doses. You can see that reaching here was difficult because we are talking of $13 per child. Getting up there, it will be very, very difficult.

The cost for applying the vaccine is around 15 to $20. We will have many problems getting the newer vaccines into the countries.

This is only to show vaccine coverage, as you can see, divided by the regions. The problems are mainly in Africa where there are many countries that have very low coverages, but also some Asian countries.

So what do we need? We need vaccines. We can identify easily what kind of vaccines we need. They have to be safe and effective. We are trying to get multi-antigenic and multi-component, so every time we have access to a child, we can deliver as many antigens as possible.

Stability is a problem because of the need for cold-chain, because of transportation difficultie and delivery systems, etc. We need also an easy delivery methodologies. Injections, we know, is a problem. Oral delivery would be ideal, as it may help in developing mucosal immunity, that would be the greatest benefit. As we know, most of the infections start through the adhesion of microorganisms, so if we can stop this, we can avoid then the infection. Ideal vaccines should require easy storage and transportation. And the most important component, they have to be affordable.

What do we have? We have many new tools available, and many groups and lots of research is being conducted trying to solve this problems. We have a better understanding of pathogens, pathogenicity and virulence. We are getting better understanding of the host immune system.

We can express antigens. We can develop expression libraries for many pathogens. We can produce proteins from

recombinant systems. New vectors can be developed to make live vaccines, genetically modified proteins or detoxified toxins.

We have technology to conjugate antigens to carriers. We are looking at new delivery systems, adjuvants, trying to develop technologies that can stabilize better the antigens. And again, we have nucleic acid vaccines.

Now we have the appearance of plants. We have been in contact with plant immunization since the very beginning, we have been genetically manipulating and working with plants. Plant extracts have been used in medicine for centuries. So we may use them now to generate recombinant antigens, extract them and later utilize them.

One other new concept -- and that's why I'm here to learn from you -- is to use the whole fruits or plants as the new delivery system for an incorporated antigen.

This cartoon is my simplistic view of how I see the plant vaccines, and I hope that at the end of this seminar, I will be able to prepare for the next time a much nicer cartoon.

So we can have the whole fruit, probably with a label, saying something like a therapeutic indication, one banana will provide active immunization against so many diseases. Take one every two days or something like that. It could also come as a processed food, in a jar, like baby food, and a very important industry for processing foods already exists that can facilitate the production and delivery. They would come with an insert as for any other biological pharmaceutical drug.

We can see that there are still many problems, particularly regulatory, because now we have a new group of products, fruit product and biologics. They have to be regulated according to food, but also, as biologics. So there are many questions that we will have to address.

That is one other reason that we are here to listen to regulators, how they are planning to define and measure stability, consistency, potency of these kind of products, and how are they going to define and implement the GMP?

I am almost done, but there is still something important that we have to consider, and it's what I tried to label as ethical and social issues. We are dealing with new technologies. The new technology like DNA base technology have produced for us many new and exciting discoveries, new drugs, new medicines, new vaccines, new assays, new diagnostics.

We are using terms that perhaps the public in general can get frightened. I mean I am still concerned when someone talks about nuclear medicine, I kind of get scared.

So we have to be aware that we might be using some terms, and we have to be able to explain to the public what we are talking about. The public understanding of science or of this technology will be very important if you want to have a benefit out of them because misinterpretation can lead us to no good or usable product at the end.

I think we have to understand benefits and possible risks, and we should be able to communicate them in advance before it becomes a major problem.

And we have to be able to try to communicate to the public all this excitement and all these discoveries of the scientific advances. And I guess something that it's important because that's what we are discussing about, plant biologics considering them as an alternative to have affordable vaccines for the developing world.

So I think that's important to consider, to focus on.

I mean if we end up with a product that is as expensive as the product that we have available, they will never reach the developing world. And if they are not going to make a major impact in the developing world, we have lost our focus.

And then I would like to finish with this sentence. The use of vaccinations to its full potential and to reduce inequities within the countries and around the world, we must not only cope with the technology challenges but also manage the political and communication issues much better.

And I would like to thank you for your attention, and now let's go to business.

LOUISE HENDERSON: Are there any questions for Dr. DiFabio? Okay. Well, I think Dr. DiFabio really set the stage for this meeting. There is tremendous potential with plant technologies to solve many problems we face today in safety, in delivery, in cost, and to meet the needs for diseases that are not well controlled by products that are currently available.

Those of us in the veterinary field also recognize that not only do our vaccines prevent animal suffering, but they also have a huge impact on a human condition, both in preventing zoonotic diseases and in preventing famines and loss of economic power for both developed and underdeveloped countries.

Certainly famines have been caused by veterinary diseases that have had huge impacts on the human population. I think as we develop new products, it's important that we need to recognizeboth the potential and the risk associated with this, and hopefully this meeting will set that. So thank you, Dr. DiFabio. That really set the stage for this meeting.

The seminar is designed to provide information, and it's going to take us from the concept to product. We're going to try and cover the whole basis, so as you know, we're not going to be able to get real deep in depth, but we hope that we will have a good overview for all of you.

And to start that off, the first session we will talk about expression systems. I'm excited to introduce Dr. Guy Cardineau. Dr. Cardineau is a molecular and cellular biologist. He's worked in the field of agricultural biotechnology for over 16 years.

He has patents pending in and patents that have been granted in plant sciences, including three broad-based patents describing production of vaccines in transgenic plants. He's now with Dow AgroSciences, and he will be presenting a talk on the Basics of Transgenic Plants 101 which should serve as a foundation for the rest of the talks at this seminar.

GUY CARDINEAU: Thank you, Dr. Henderson. Okay. I've been asked by the organizers to sort of describe ag biotech, so we called it Ag Biotech 101. What I'm going to try to do, I talked to Jim White last night and said I wasn't sure if I should start with 1800 B.C. when yeast was first used in fermentation, but I figured that was a little bit too far back. So we're going to focus on the last two decades and -- if I can get my machine to work. There we go.

Starting in 1983, actually. The first report of transgenic plant cells was made at the Miami Winter Symposium in January of 1983 by three groups: a group from Monsanto, a group from Max Planck and Ghent, and a group from Washington University.

And they all described the use of Agrobacterium mediated transformation to move bacterial antibiotic-resistant genes into plant cells and were able to select for stable transformation of those cells based on resistance to the antibiotics.

The first transgenic plant gene --that is, the gene from a plant source – was described about five months later.

Now, this is a cartoon that describes the mechanism of Agrobacterium transformation, and I don't expect you to follow this. It's a very busy and involved cartoon, and the reason for that is that Agrobacterium transformation of plant cells is a very involved process. We still don't really understand it completely today. But I'll try to describe it a little bit more easily.

Essentially, Agrobacterium tumefaciens is a soil bacterium. It carries a very large plasmid called the Ti plasmid for tumor inducing. It's about 200,000 base pairs. And on that plasmid is a region of DNA called the TDNA or transfer DNA that is actually transferred into the plant cell.

Now, a philosophical question you may ask is, is it plant DNA, or is it bacterial DNA? Is it eucaryotic or prokaryotic? And I think that's a reasonable question, in that the genes work in the plants, so the regulatory signals function in the plants. And they don't normally work in the bacterium.

On that plasmid -- in that TDNA region there are a number of different genes that different code for opines, which are sort of aberrant amino acids that the Agrobacterium can use as a carbon and nitrogen source, and there are also genes that code for plant hormones.

Those hormones result in the production of tumors, and in fact, this system was used as a study system for mammalian cancers for quite some time.

Now, here's a cartoon that essentially describes what happened. Naturally, Agrobacterium infects at a wound site, so when a plant seed germinates and breaks through the ground, sometimes there are tears in the stem segments, and Agrobacterium is attracted to the wound exudates and will infect and then transfer parts of its DNA.

Now, there are chromosomal genes as well as plasmid genes that are involved in this, and there's an attachment phenomenon and then actual transfer, much like conjugation of bacterial cells.

So here's a picture of an actual tumor on a plant. This is not a transgenic plant or anything. This is a regular infection. But what scientists considered doing and were successful in doing was to replace the TDNA with genes or DNA of interest. And so they took an expression cassette, and by that I mean a promoter that would operate in the plant cell, and these early constructs actually used promoters from TDNA or viral promoters. They used a structural gene. They used a three-prime sequence which

would allow for addition of Poly A residues to make a mature message.

And in some cases you might have other things in there, an enhancer or signal sequence or whatever. I'm sure maybe Doug Russell is going to tell us a little bit more about that when we get into the induced expression and constitutive expression talk.

But essentially, this is the way the TDNA is set up, and you have virulence genes, there are eight operons that are involved in mobilizing the TDNA into the plant cell. The TDNA exists in one section, there.

Now, when these original constructs were made, the original conception was to make cointegrate plasmids., So they took a piece of DNA they were interested in. They put on an E. coli plasmid. They associated the DNA or gene cassette of interest with pieces of DNA that were parts of the TDNA and then conjugated that into Agrobacterium and allowed for homologous recombination to insert that DNA of interest into the TDNA region.

In some cases the vectors were armed. That is, they had the oncogenes -- the tumor or hormones genes. In some cases they were disarmed. The original vectors were built this way. It's fairly unwieldy, and another set of vectors were developed called binary vectors.

It was discovered that the virulence region which mediates the transfer of DNA into the plant cell could be removed from the larger plasmid and could be used separately and that the virulence functions would act in trans to mobilize the TDNA of the smaller plasmid.

Well, this was a big benefit to us because a 200,000 base pair plasmid is a little bit unwieldy to work with. So now you can use a basic E. coli-size plasmid. You have to have a replication origin that works in both Agrobacterium and E. coli, but it's fairly easy to make these.

And these plasmids have been developed quite extensively, and there's a whole range of these things around, but this is sort of a schematic of what one might look like using kanamycin resistance as a determinant.

There's a right border and a left border. Those are 23 base repeats. There're three nucleotides that are different between those. They actually form the borders, so the direction of transfer is from right to left. So you can orient your genes in there, and it will be transferred, generally thought of as a single stranded DNA molecule coated with proteins from the virulence region, into the

plant cell and somehow get into the nucleus and are incorporated into the genome. Okay.

In 1986 we have the first field-test in transgenic plants, and it was a Bt tobacco. This was done by PGS, Plant Genetic Systems, and Rohm and Haas, and these were native Bt genes. This was done using an Agrobacterium mediated transformation system.

One of the problems with hat Agrobacterium, though, is that at that time, anyway, lots of people were trying to work on transformation of monocots, and it didn't seem to work. The host range of Agrobacterium is principally dicots. Obviously, corn, rice, wheat, other monocots are very important, and so many folks were trying to work to develop a way to address putting genes into these sorts of plant cells.

Well, in 1985 or so, Mike Fromm and Ginny Walbot developed a technique for plants called electroporation. So in that technique you mix DNA and protoplasts (that are plant cells with cell walls removed) in a buffered medium, put it in a cuvet that has electrodes, apply an electrical charge to that. It opens the pores or a pore in the membrane of the protoplast. The DNA is taken up and makes its way to the nucleus, and they demonstrated stable transformation of maize protoplasts.

The problem was it's very difficult to regenerate plants from PROTOPLASTs. So this was a technique that evolved, and today there are folks that have been able to make whole regenerated plants, stable transformants, using this sort of a technique because the technologies have evolved to regenerate plants from PROTOPLASTS. This is just a picture of some of the sort of equipment that's used to do this sort of work.

Now, this is the device called a particle gun. It was developed by John Sanford at Cornell, and it really revolutionized transformation of plant cells. Ingo Potrykus once described this device at an international meeting as the curiously American way of transforming plant cells. Shoot them. Sort of the Dirty Harry approach, I guess.

Anyway, the original gun actually was a gun. It used a .22 caliber blank cartridge as a mode of force to fire projectiles which were gold or tungsten particles, micron-size beads that had been coated with DNA, into plant cells.

This device here in this photograph actually uses helium. There are various ways you can derive the force: helium, electrical discharge. And this actually is a fairly well controlled device.

There's a cartoon over on the far side in which you can see that you have a screen. You coat your beads. You put them on this sort of carrier membrane. When the mode of force is applied, it hits a screen, and the particles pass through that and hit your target.

There are ways that you can alter the intensity so you can actually impact the depth in the tissues that you drive these beads, and amazingly enough, plant cells do survive when they've been fired at like this. Some of them die but not all of them.

One of the disadvantages of this system is you tend to put in lots of copies of genes, and that can sometimes be a difficulty because there's a phenomenon called transinactivation or gene silencing that's impacted by multiple copies of genes. But this is a very efficient way to transform plants, and many plants that are in the marketplace now, transgenic plants, were developed using this sort of a technology.

The technology has advanced so much that you can actually have a hand-held-type gun. It's like a nail gun, in fact, and it will work also to transform plants by just simply shooting leaf tissues.

Now, another method was developed around 1990 or so, probably late '80s early '90s, originally developed in insect cells, and it's using something called silicone carbide whiskers. These are little speres. They're about a micron in diameter, but they're quite long, 500 microns perhaps.

And using this system, instead of coating these speres with the DNA material, you actually put them in a solution of DNA, plant cells, and these little speres, and you get impaled plant cells.

The way this is done is not by focusing these materials at a target, but you can use a vortex, or actually, one of the best devices is something called a wiggle bug or something like that that's an amalgam maker the dentists would use. It just simply shakes the material like that.

In this case when these little silicone whiskers go into the cells, they actually drag the DNA with it. This has also been successfully used by a number of groups. In fact, Dow AgroSciences is using this procedure right now.

In about the '94, '95, '96 time frame, the first transgenic plants hit the marketplace. The most famous would be in 1994, Flavor Saver Tomato. It contained the polygalacturonase gene, put in backwards, in an anti-sense

orientation. They were trying to impact the solid -- or how do I want to say this? -- the ability of the plant to withstand rough handling in early stages.

And I was really curious why it was called Flavor Saver Tomato because I don't really think it imparted a flavor characteristic as much as it allowed the plant to withstand picking at a later stage, and it appeared to be riper.

In '95 we came out with Round-Up Ready soybeans, insect-resistant corn, and insect-resistant cotton. Now, the corn and the soybeans were all developed using particle bombardment. The cotton was derived using Agrobacterium mediated transformation. Insect-resistant genes are from Bt, Bacillus thuringensis, and these are some of the products that are currently on the market.

So we see here we have Bt insect-resistant corn, cotton, and potatoes. We have herbicide resistance in soybeans, canola, cotton, and corn; delayed ripening in tomatoes. In addition to Flavor Saver, which is no longer in the market. DNA Plant Technologies has a tomato called Endless Summer, I think. And ICI in England had a tomato used in processing, and you could go to grocery store to buy this -- this was a tomato paste, and I think they've recently pulled that from the market due to the uproar in England over genetically modified organisms.

And then the last is virus-resistant vegetables. They're also available in the marketplace. A lot of these things have been around for a fairly long time now.

And the technology works. This is some early Bt insect-resistant corn. You can see on the panel on your left control corn leaves and transformed corn leaves on the lower part, significant damage difference between those.

And the panel on your right are stalks. They're split stalks. The stalks on the left-hand side are transformed. The ones on the right-hand side show the damage of European corn borer.

It's anticipated that with the development of these insect-resistant plants, within a fairly short time we may be able to eliminate or drastically reduce most of the agricultural insecticides that are currently used in corn. And this is a projection of where we think the industry is going, and there are, of course, a lot of players in here: Dow AgroScience, Monsanto, Pioneer/Du Pont, Agrevo, et cetera.

But coming up here in 2002, we expect to be able to target corn rootworm, which is a big problem, and using some fairly hefty insecticides for fumigating fields.

Bt cotton is also a very popular product. About 50 percent of the cotton grown in the United States is insect resistant by virtue of this technology, and this is the projection of how the market is anticipated to go. You can see that it's anticipated it's going to spread internationally as well.

And if we look at glyphosate tolerant soybeans, we can see, again, that it's anticipated that over 80 percent of global soybeans will be glyphosate tolerant in about eight years. That, of course, depends on whether or not the technology is allowed to continue.

All right. So in 1999 we have had extensive debate in the U.S. regarding GMOs, and I don't know where 2000 and beyond are going to take us.

But I think part of the issue here is that those genes and products are what we've been working on -- and now we're starting to look at some new things, and that's the purpose of this meeting today. We're looking at the development of output traits. So input traits are those things we put in the plant that the farmer can use to improve growth of the plant: insect resistance, herbicide resistance, disease resistance, abiotic stress tolerance, things of that nature.

Output traits would be anything a plant can make, and so a projection for revenue generation over time indicates that by the year 2010, this is a $200 billion potential marketplace; all kinds of products that can be made in plants.

Now, historically, we've been limited in our ability to put genes in plants, usually one or two genes or a very few number. We've used known genes like Bt. Herbicide biochemistry is fairly well understood, so herbicide resistance was an obvious target.

We're now moving into areas that are going to become a little bit more complicated, but I think we have new sets of tools that are going to allow us to do that. One of the things that we'll hear about later is antibody expression in plants.

Secretory antibodies are fairly complicated. There are four genes to make a secretory antibody: a heavy chain, a light chain, a J chain, and a secretory component.

It's possible to put all those genes in together on one construct or multiple constructs or in different plants

and cross them. So there's a variety of ways you can address these issues.

And we have another tool that we can use, and that's viral delivery as opposed to using transgenes. Allen Miller is going to speak after me about viruses, and we'll have Barry Holtz from LSB who is going to talk later about some work regarding that issue as well.

There were some differences between using transgenes versus using viruses. With transgenes in this case where the carrier was TDNA we moved the gene into the plant cell. It gets in the nucleus. It's incorporated in the genome. It takes a long time to do this.

With viruses you can use a single-stranded RNA virus and infect the cells and get spread in the plant and make a large amount of protein in a fairly short amount of time. There may be issues with regard to stability. You can store things in seeds for a longer period of time. In the case of viruses, you're going to do that in green tissue, and also there may be some limits on the size of inserts. I'm sure we'll hear more about that later.

But we're now, I think, at a point where we can handle multiple genes in plants, and we can better control expression. There are more complex genes, and we're moving into other options or opportunities.

If we look at this sort of a chart that indicates value versus volume, we can see that the food plants are the high volume, low value sort of activity, and the pharmacological things are the more low volume but high value activities.

So we can touch on a few of those. There are some products that are currently on the marketplace that are nontransgenic that were produced by mutation breeding, but they address this sort of issue at that front end: a high oleic sunflower, high oleic peanuts, high oleic canola, plus high oleic soy oil. These are all available now.

We can look at higher vitamin content, reduced anti-nutritionals, low sat fats, and increased nutraceutical content, improvedamino acid composition. People have been working on improved amino acid composition of seeds for almost 20 years now, and I think we're finally at a point where we may be able to actually address these things.

If we move into the specialty chemicals in the polymers area, right now Dow Chemical is working with Cargill producing polylactic acid from corn. They're using that to plastic that is currently being used to make yogurt cups, and they can use it to make fibers, so they may be

able to make clothing out of it and a wide range of things. It's a renewable resource, and it's a very attractive possible product.

In the oils area there are all kinds of oils that can be used for both industrial and for food applications. Long-chain polyunsaturated fatty acids, for instance, fall under the essential fatty acid area at the top there, and I think there was a recent announcement by the government that they were recommending addition of these things to infant formulas.

Okay. We move to nutritional supplements. Nutrition is a huge field. We can go from vitamins to minerals. We can get into the nutraceuticals area. I don't want to spend a lot of time on this slide, but obviously, there's a whole range of products that could be made in plants and a lot of things which are plant-derived products but which we could make in other plants that we can grow in larger volumes and produce more of these materials.

And then we get to the molecular "pharming", the high value protein. If we look at 1996, this is a cartoon of a pie chart that indicates biotech products in clinical trials: 78 antibody products, 62 vaccines products.

Most of these products are proteins, and we can produce proteins in plants. So plants make a very attractive source for producing these things in high volumes with low cost.

If we look at the anticipated market over the next 10 years, we think that both the antibody and the vaccine markets are going to grow, and one of the ways I think we can address this growth is by expressing these molecules in plants, again, reducing cost.

Okay. So in summary then, this is actually still a fairly young industry. It's been 17 years since the first report of transgenic plants, but there really are very few products in the marketplace, and we're now just on the verge of producing those products that will have more direct benefit to the consumer.

There are all kinds of opportunities, I think, from the low-volume high-value pharmaceutical products all the way to the high-volume low-value food products. And there are a number of organizations that are involved in doing this, both industrial organizations as well as academic and national labs, so there's a whole range of people that are trying to work on solving these problems.

I think this sums it up for me, and that is that there's now a confluence of a variety of factors, I think,

that's going to allow us to move forward to use this technology and develop a whole range of products, including vaccines and antibodies and things of that nature.

Thank you.

LOUISE HENDERSON: Are there any quick questions for Dr. Cardineau? I think it's really exciting to think about all of the possibilities when you look at basic technologies being produced, both antigens and antibodies. It's tremendous potential for us.

Next we're going to hear from Dr. Allen Miller. Dr. Allen Miller has a Ph.D. in Molecular Biology from the University of Wisconsin, and he's done research on replication of brome mosaic virus RNA in vitro, and he is now on faculty of the Plant Pathology Department here at Iowa State and is an affiliated member of the department of Biochemistry, Biophysics, and Molecular Biology. And he also teaches as part of the graduate a course in molecular virology in the Veterinary Microbiology and Preventative Medicine Department here at Iowa State.

Dr. Miller's presentation will focus on engineered viruses and their use in expressing biological molecules in plants.

ALLEN MILLER: Well, I'm pleased to be here, and I'm really more of a basic molecular virologist, and I really haven't kept up on all the latest advances in the industry, so I apologize if I'm not quite there in terms of examples of genes being expressed by viruses, but what I am going to be talking about is something I do know more about, which is just the fundamental mechanisms of virus gene expression and how they relate to expressing useful genes from viruses.

So viruses are basically -- from the point of view of interest to this meeting, they're extremely efficient machines at producing huge amounts of protein. An example here that you probably are all familiar with is tobacco mosaic virus, and this can grow in such high levels that it forms crystalline arrays in plant cells, up to 10 percent of total plant protein. And an amazing thing is that the plant can live. It can survive.

So with viruses like these, what's amazing about them is not that they make plants sick, but it's how do they not make them sicker, because evolution has favored that they don't kill the plant. An example shown here, this plant might be just filled with virus. It looks a little unusual but it's certainly alive.

And what I am going to talk about today is first I'm going to give an overview of some of the advantages and

disadvantages that I could think of of using viruses as expression vectors, and then I'll give examples of some virus gene expression strategies and how they're different than the way normal genes are expressed and how they can be exploited and have been exploited.

And then if I have time, I'll get into some of the real details of what we know and still need to know about virus gene expression to really be able to manipulate them.

So initially, here are just some of the advantages of viruses versus what we heard about with the stable transformation. First of all, it's really easy to get the virus in. With ones like TMV, most of the ones that are being used, you can just rub the virus on the plant or spray it on, and it delivers. It transforms the plant. Basically, in a transient way, it delivers genes.

And you can get extremely high levels of gene expression, as I already mentioned. I think frequently with TMV, they get up to maybe over 1 percent, 2 percent of total plant protein. The expression is transient.

So if you have some protein that for whatever reason the plant doesn't like, you can grow a plant, infect it, express your protein. If the plant gets sick, you don't care because you've got enough protein made to harvest it.

It's easy -- if the protein is actually fused to the viral coat protein, which is the case for a lot of the vaccine constructs. You can just purify the virus, and plant viruses are very easy to purify, and that's why many are more structurally well characterized than animal viruses.

And plant virus genomes are small, generally smaller than animal virus genomes, usually under 10 kilobases, so they can be cloned into regular plasmids and manipulated, and they're easy to manipulate with that regard. But getting one that's actually functional isn't so easy.

So that leads to some of the disadvantages. Viral genomes, because they are small, they're very densely packed with regulatory signals, coding regions. There's often overlapping genes or subgenomic RNA promoters that overlap with coding regions. So it's a little harder probably to mess with a viral sequence certainly than it is with a plant genome sequence where there's lots of flexibility.

You don't have nice seeds that have a gene. You have to inoculate each time you want expression. With the virus, there may be some kind of risks if you're spraying virus out in the field if it actually happens to be a pathogen. You don’t want it to get out.

And in general, I'll be talking mostly about RNA viruses or complete RNA viruses, and that's the vast majority of plant viruses and animal viruses, for that matter.

So RNA replication is much lower fidelity than DNA replication, much more error prone. So the genes of interest might mutate, and there have been problems in the past with instability.

In fact, in the 1980s a lot of people said RNA viruses wouldn't work as vectors because they're not stable enough. But this could actually be an advantage because if you accidentally do create some pathogen that your gene of interest actually makes a virus more severe, well, probably it would not last long in the nature because it would mutate, and there would be no selective advantage to having those traits.

Another problem, of course, is that plant viruses usually make plants sick. They do have some symptoms. And it would be nice to be able to disarm those, to know how to disarm them the way Agrobacterium has been disarmed. And another thing is the host range. You need to have a host that your virus of interest can infect.

So I'm going to summarize a couple different gene expression strategies now and how they've been used. One example is in which, okay. A virus has an RNA genome, and the whole life cycle for most RNA viruses occurs completely in the cytoplasm, so they can't take advantage of all the transcriptional control mechanisms that are used for normal host genes.

So instead, they've evolved other mechanisms for regulating gene expression, sort of post-transcriptional. And another thing you care about is mRNA. Generally one mRNA encodes one protein. So an RNA genome that requires several proteins must have ways of expressing them.

I'll describe several ways have evolved, one of which is to produce what we call a poly-protein which is the whole viral genome. All the proteins that are needed are actually encoded in one giant gene which gets translated into what's called a poly-protein.

And examples of these viruses include the very large poty virus group, and como viruses which includes cowpea mosaic virus, which I'll be talking about. And for you animal virologists, the picornaviruses Foot and Mouth Disease, polio. Many common viruses, common cold viruses, also use this strategy.

So what we have here is the first translation product, the giant protein -- and I show here an example of how one might insert a gene of interest. It's just fused right in there.

And then what happens is there's a series of very well orchestrated proteolytic cleavages. They're built-in proteases, and they also use host proteases. They cleave at very specific sites.

So now you're starting to get the individual proteins, and these intermediates may have some functions, and then there's additional proteolytic cleavage to get the final products including, for example, the viral coat proteins that replicate and so on.

And if you have a good understanding of these proteolytic cleavage sites, you can insert the protein of interest which can either be cleaved out to be totally independent or perhaps fused to something like the viral coat protein. And this has been done in George Lomonossoff's lab with cowpea mosaic virus in which -- actually, I should give him credit. This is from his Web page. I just found this little image.

And basically, there's two viral RNAs, and they've been cloned in the plasmids. So you have two plasmids. One has all the genes required for RNA replication. The other one has the structural genes, the coat protein genes.

Then these are manipulated to introduce the foreign gene of interest. In this case it's a very small amino acid sequence that's antigenic to whatever human or animal virus is of interest.

And then in all these examples I'll be talking about, you manipulate things on regular plasmids in E. coli, then make the infectious RNA. You take advantage of a bacteriophage promoter, which really isn't shown here -- maybe it's right there. I guess it's not here – to transcribe the viral RNA, and then the RNA is infectious.

And this can be done very efficiently and very easily in vitro in cell-free system using a bacteriophage promoter here that doesn't function in the plant but only is used in vitro to produce the viral RNA.

So then the plants are infected, shown here, and the virus propagates and drags along this little extra antigen here on the coat protein.

Purified virus, as I said before, is very straightforward and end up with a -- let's see. I think

these spin here. This is from Jean-Yves Sgro's Website in Madison. They have a lot of these neat pictures.

But this shows a related como virus, and you can see these protrusions here in white -- well, this is where the Lomonossoff and colleagues have put various viral epitopes from animal viruses so they're nice and protruding right out there and available. And these can be injected into whatever organism you want to immunize.

And there's two here because it's actually a bipartite virus because it requires two components. And this also shows an important thing you need to know. To get this to work you have to have a good understanding of the virus structure and exactly where the surface parts of the viral coat protein are.

Okay. Here's some examples that I'm certain are out of date, and I'm sure there are many other published examples that have produced epitopes from HIV, Human Rhinovirus 14, and thenin one case where they took a parvovirus, and they found an antigen that would actually conform to three different parvoviruses, including canine parvovirus, a mink enteritis virus, and I think a feline virus. So those are some examples.

Now, another virus that is well used and you'll hear more about from Barry Holtz is tobacco mosaic virus, and this uses a different strategy. Here you have several genes on one viral RNA. There's only one genomic RNA for the genome, but only the first open reading frame -- there's actually a second one here that I'll show you later -- is translated.

So the infection initiates. This is the only protein that is made. And it includes the viral replicase, so it can replicate the viral RNA, and in that process it produces subgenomic RNA by RNA-templated transcription. Now, this is the message for this viral protein.

And then if you have an interesting foreign gene that's been inserted, so this is the extra one that would be expressed. And then additional viral proteins would actually be the viral coat protein.

So here the strategy is to insert the messenger RNA and have this transcript so you have a transcriptional regulatory step in the expression. And that has allowed expression of -- I should just point out this allows expression of a protein that's not fused to any coat protein, so that can be an advantage because then you don't have to worry about attached proteins or, you know, amino acids that may affect function. You get a pure unadulterated protein.

However, then you have to purify it. You can't just purify a virus. And actually, some Japanese group, at least, has taken the viral coat protein which I didn't label here and fused on an interesting protein. So it was able to be coat purified with the virus.

And I'll just show you, again, the virus structure here, and it also reveals some advantages here. This shows that there are many, many subunits of the viral protein, so if you have an interesting epitope or something fused to this, you could have many, many sites, you know, studied on the outside.

And again, it shows you have to really have a good understanding of the viral structure to know where to fuse these to the coat protein.

And then this demonstrates another important feature of tobacco mosaic virus, and you know, several different kinds of virus, Potato Virus X. And that is an advantage to be a rod-shaped virus because you can insert DNA or RNA and make the whole viral genome bigger like the example this shows, actually.

And what happens is the RNA is packaged right inside this cylinder, kind of spirals around in there. And the longer your RNA, the bigger the particle. And there's theoretically not much of a limit whereas in those spherical particles that I showed before, there's a definite size limit on the size of the RNA you can get in there, so that's kind of the disadvantage with spherical viruses.

So here are some examples in the literature. I mentioned this TMV coat protein fusion with angiotensin II converting enzyme inhibitor and angiotensin protein.

And then examples of proteins expressed from a free -- it's a separate, completely free protein. The extra subgenomic RNA encodes ALPHA trichosanthin and single-chain epitopes that are characteristic of lymphoma cells that can allow for a vaccine against certain lymphomas, and you'll probably hear more about these later today, again, from Large Scale Biology.

So now a third gene expression strategy involves multiple RNAs, so rather than having a lot of the genes on one big RNA or having a polyprotein virus such as a poty virus, they just have several RNAs. They have four RNAs. They also produce a subgenomic RNA here. Put all four RNAs in the viral particle, and these two encode the replication functions so that products of these RNAs can replicate their own RNAs as well as these other RNAs that encode the protein

that allows the virus to move in the plant and the viral coat protein.

So although this is a spherical virus, it may be possible here to -- because these replicate in trans -- in other words, it replicates other molecules. You might just be able to build another RNA and have an extra fifth RNA or whatever that has the replication origins from the ends and is replicated by the products of this.

And this would be analogous to defective interfering RNAs that many of you are probably aware of in animal viruses. Actually, historically brome mosaic virus, I think, was the first virus that was actually used as a vector --first RNA virus to produce proteins in eucaryotes, certainly in plants, and that was the way Roy French and Paul Ahlquist in about 1984 or '85 expressed a reporter gene in which they replaced the coat protein with a CAT gene.

So to summarize, what are some good properties of a plant virus vector? These are ones I can think of. It should be mechanically transmissible, easy to inoculate and especially in doing large field experiments that we'll hear about later.

But you don't want it to be picked up by some insect vector and transmitted all over, so it would be nice to have one that is not very efficiently or not at all transmitted by any vectors that might be around.

You want one that will tolerate insertions. As I mentioned, rod-shaped particles can tolerate longer RNA, such as TMV, tobacco mosaic virus. Jim Carrington has been doing some things with tobacco etch virus, and Potato Virus X also been widely used. They also have rod-shaped virus particles.

Or perhaps multiple RNA viruses so you can have an extra RNA in its own particle. And cowpea mosaic virus has two particles, and as I mentioned, brome mosaic virus.

You want a relatively small genome for ease if it's too small, it might not tolerate much change, and that's been a problem trying to figure out how to manipulate these viral genomes without accidentally knocking out some function that you didn't know was there.

Obviously, we don't want viruses that kill their plants or make them really, really sick. So that's a big area, I think, that could be studied, is how viruses -- and is being studied -- how they make plants sick and how to manipulate that.

You want high-level expression, I think, for the most part, and if you could manipulate host range, that would also be very useful for both expression in desired host as well as preventing escape into the host you don't wantit to replicate in.

So in the next few minutes, I'll talk about focus on TMV and just give an example of all the stuff you have to know or you have to be aware of. Maybe you can get lucky and not know about it and get away with it, but to make an infectious clone -- just make an infectious clone of the virus is not trivial. Often there's a lot of variability in the population, and the particular one you clone isn't really very viable, and it has been sort of supported by other RNA molecules.

You have to remember that viruses are like thousand-ploid or more. There's thousands of copies of a viral genome in each cell, so what you need is a certain percentage of those to actually encode functional proteins, and the rest could be going along for the ride.

So it's sometimes difficult to clone an actual infectious RNA, but then once you do, you have to be aware of how you can manipulate it without knocking out controls.

Using tobacco mosaic virus as an example, there's a lot of complicated translational control, and some signals are shown here. The five-prime untranslated regions is actually a very efficient translation enhancer sequence called omega, and this is actually used in a lot of stabley transformed plants. It is just a way of getting high gene expression independent of any virus.

I should mention there's actually another trick. Viruses use a lot of kind of translational tricks to express extra genes that I am not really going into here too much, but one example is in TMV. The polymerase is actually only translated by a read-through of the stop codon. So you get a lot of translation of this protein. Then there's a programmed read-through caused by certain flanking sequences that allow the ploymerase to be made.

And the group I referred to earlier, they actually took advantage of this and took this signal and stuck it on the coat protein so they could make a read-through on the coat.

And then there's things that were 10 years ago totally unsuspected. There's a thing called a PSEUDOKNOT-RICH DOMAIN down in here that's actually required for translation. This virus has no Poly A tail here as they were supposed to have, and somehow while the mechanism is

not clear, this domain can substitute for that. And I'll show some examples here.

Sort of summarizing some translation sequences, barley yellow dwarf virus, which is actually the virus I spent most of my time on. There's an omega sequence, and then there's a PSEUDOknot-RICH DOMAIN, which as I mentioned is actually required for translation. Poly A tails interact somehow via various translation factors with the five prime end, and with the cap they all form kind of a circular mRNA, and somehow these can substitute for the PSEUDOknot when you have a stem loop in the RNA and then the loop is base-paired to another region that's downstream. So the RNA goes like this, like that, like that. And that doesn't look very good in two dimensions. There's a whole series of these, actually, that's required for translation of TMV. And I'll show on the next slide a really neat picture of a three-dimensional structure of one of these.

And that indicates other viruses don't have a five-prime cap. And so they have sequences that allow translation, even though there's no cap. So we call it cap independent translation.

Five-prime caps are modifications that are on all eucaryotic mRNAs except certain viral ones, and they're required for translation except for certain viruses.

So this is a secondary structure of the one in barley yellow dwarf virus, and this, too, is actually being used or potentially being used as a tool for high-level gene expression in transgenic plants, and it allows using promoters that don't necessarily produce capped RNA.

So what's a Pseudoknot look like in three dimensions? This is a nice picture showing one from Beet Western Yellow virus, and you can see this is how the RNA gets all contorted, and there's lots of NON-WATSON-CRICK types of interactions here, and it just gives you an example of the complexity of the regulatory sequences involved in RNA viruses.

And controlling that read-through, as I mentioned before, Jim Skuzeski found that around the stop codon you need an A followed by this consensus motif, and you get about a 1 percent read-through. And that's actually been exploited. Again, barley yellow dwarf virus also does it a totally different way in which there's this motif here. My microphone is going out.

There's this double C motif and then something located over 700 bases downstream that allows read-through of that stop code. That means that the -- I should back up. It translates the first protein, and then most of them stop.

Some of them keep going and add a little extension on or a big extension. And then a pseudoknot can also affect this in murine leukemia viruses.

So the point is there's a whole lot of different ways that viruses have evolved to regulate translation, and then we have to think about the same thing all over again with transcription RNA templated transcription.

With TMV the wild-type virus has the movement protein here and the coat protein here, each with its own subgenomic RNA promoter. In early work by Bill Dawson and others, they thought let's add an extra gene, so they then duplicated the coat protein promoter for a foreign gene, but it was quickly lost because of homologous recombination. Probably it would have homologous blocks of identical sequence within the same viral genome.

And another problem you have with duplicate promoters is the downstream ones are the most active, and the upstream ones are much weaker. So what was done was they took the subgenomic promoter from a different virus like odontoglossum Ring Spot or tomato mosaic virus, and it's different enough that it won't homologously recombine, but it's similar enough that the viral replicase will recognize that promoter, and that allows for expression of the foreign gene. So that's another example of the complexity.

So in summary, there's a whole lot of complicated set of viral sequence elements. Still even with TMV, there's a lot of work to be done to really understand what's going on that would help optimize viral general expression.

And finally, you name it. Here's some examples of what I think could be discussed for further research. It's more a lot of understanding of viral structure, and you want to understand how you want high-fidelity replication of your genes. You want your genome to not be spreading out the environment, yet you want it stable enough for your own purposes. And again, I sort of mentioned all these other points before, and I think with that I'll stop and take any questions.

LOUISE HENDERSON: Thank you, Dr. Miller. I think for those of us new to the plant world, it didn't take very long to move from Basic 101 Plant Transgenics into some really cool science that is pretty complex.

So we're going to continue on that note. Dr. Douglas Russell will talk next. He is with Integrated Protein Technologies for the Monsanto unit. His research work for his Ph.D. degree was in plant and protein synthesis in corn and during plant stress and development.

He is going to talk to us today about constitutive or induced tissue specific expression, which we think is a very valuable method of getting around some of the environmental concerns that will be present for plants that are grown in fields versus those that will be grown in controlled situations such as greenhouses.

Dr. Russell.

DOUGLAS RUSSELL: I want to thank the organizers for giving me the opportunity to share some of this technology in plant expression of biologics. I think it has a real chance to impact health care in ways the traditional systems can't, and to do that to be successful, we're really going to need the collaboration of a lot of the different groups represented at this meeting.

The system I'll be discussing is corn, and we've worked with a few other species, and I'll discuss some of that data, both from ourselves and others.

We see corn as a very useful system. It's building off of a proven technology that Guy Cardineau discussed. And from that data base as well as our own work with biologics, we see it as a genetically stable system as well as showing product stability in terms of its expression levels and quality.

With the work we've done for clinical trials and work others have done, we see the ability to work under the GMP-type controls necessary for biologics can be done in plants. But we don't simply want to replace a traditional system but want to try to add some new value.

And I think one of the big areas where we'll help is addressing capacity limits with traditional systems when we start thinking about needs of, as mentioned earlier, cheaper therapeutics or vaccines or large-volume uses, things for chronic therapies.

The transformation system we generally use is a biolistics approach. Agrobacterium has been used in corn as well. The basic approach is to take an ex-plant of the plant material that allows the gene to be delivered, select for those materials that have the gene inserted and then regenerate a viable plant that eventually yields normal-looking corn seed.

For transgenic corn we've seen since 1996, we discussed earlier some projects, the release of these materials commercially and at this point up to 18 million acres of corn with these transgenic traits delivering that gene in a stable manner effectively for commodity-type

businesses that don't really tolerate failure very well. They notice when you have a 5 percent yield drag.

The expression tools used for current or anticipated things in the pipeline, such as those needing leaf expression, root-specific expression, or seed expression, we can adopt some of those tools for our purposes as well.

And what's been done for the commodity purposes for the initial traits had to meet USDA and FDA end points for food and feed needs. They have to be safe. We want to reach a different end point since there will be that extra milestone of being a good biologic agent. We can adapt traditional biologics end points for our needs as well.

The general gene design, similar to a lot of other systems, requires some sort of promotor, a switch that says turn the gene on, sometimes you'll have elements that Guy was talking about, an intron, for instance.

For the gene of interest, there may be multiple gene cassettes, antibodies again requiring at least two gene cassettes, one for the heavy chain; one for the light chain. And then a separate selection gene so that you can find the cells that actually contain the gene ofinterest.

Because the gene design is constant but where it integrates can be different, you can have a whole range of expressors. These are individual transgenic events for a single gene trait. We like these (at the left of the bar garph). We like the high ones, and we'll target those for eventual buildup in later generations to develop a pure breeding genetic stock.

We can still use these materials (at the right of the bar graph, with lower expression) for initial testing of the quality of the protein that's developed in a plant. In some cases that we're working with, the protein has never been produced in any other system.

When we examine these plants with the tools that we use, it is seed specific. The advantage that you'll see later is we see high levels of expression. We know, though, that there is no leaf or pollen expression detected. In some cases that may have advantages for worker safety.

Here's a table. It's kind of large, but there's a lot more that I could have put here with various systems that are being discussed at this meeting. Some of it is from published methods; some is our own unpublished data.

And to give an idea of what may be some of the major points that are important in considering a host system, I'm going to be comparing the expression based on the

accumulation of the protein of interest relative to the total protein extracted. That ratio can be influenced by the expression of that protein accumulation of that protein and the extraction methods used.

It will vary with the host, the organ that you're targeting, the tissue targeted for collection, as well as the target protein. Different proteins accumulate differently dependent on a lot of different factors.

What we like to see is, again, is the ability to use it for multigenetic traits, things like antibodies. There's a couple of other proteins as well that need multiple genes to be successful. Sometimes you'll need helper enzymes to get effective expression to modify the protein of interest.

Three host systems discussed tobacco, used very widely for transformation, our soy experience and corn experience. The first system I'm going to talk about is actually a little different that we published recently. It's a tobacco plastid transformation system.

There's a nuclear genome, the same sort of processes you're familiar with in any eucaryotic system. But there's this other separate organelle now in plants. The plastid has its own genome. It's responsible for the green color in the plant. That organelle can now be transformed as well. It's most active in the leaf in the green tissue, and the recent publication showed expression of a biologic at about 7 percent.

It's not been completely tested yet. The system hasn't been tested yet for multigenic subunit proteins, and I think that would be a challenge when one considers things like inter-molecular disulfide bonding in such a system.

Being a leaf system, plastid expression is not very easy to store compared to a seed system. So far to date, for the biologics, a post-harvest-type system has not been tested.

Such a system would enable one to decouple the production of your biomass and the biosynthesis of your protein. That may be important in certain instances.

The more common system is a nuclear transformation with a constitutive promotor, something like 35S. People have certainly used more leaf-specific and seed-specific promotors in tobacco.

For biologics and antibodies, expression of 1 percent, sometimes a little higher, has been seen. Multi-subunit proteins, of course, have been demonstrated and the storage of the material can be challenging. Being a constitutive

promotor, a post-harvest system can't be used as effectively. And certainly there's documentation of a lot of degradation of these proteins such as antibodies in the leaf system.

The induced system will be discussed by Carole Cramer. That allows the post harvest-type decoupling, and in some instances with some proteins, you can see fairly high expression levels.

With soy we've pushed pretty hard here, and we really haven't seen the expression levels we'd like. They're fairly low, both in the leaf and in the seed, and we've tried a number of different expression systems here. All of the data shown concerns antibody expression.

For corn looking at antibodies, this particular data looks at the same coding region of the antibody as in soy, where the difference is in the gene expression element. We can see a much greater increase of product here in corn.

What I'll share further is that we can store that material which gives you a lot of flexibility in how you manage the production system different from traditional systems.

Other people have used post-harvest-type expression in seed. The system I'll be talking about is accumulation of the product during the development of the seed. Others have worked on storing the seed and then basically germinating the system and during that germination process having the product accumulate.

We've been able to take this corn production system and successfully use it in direct comparison to a traditionally produced antibody in a human clinical trial.

The antibody we made was a humanized anti-cancer antibody that could indirectly bring a toxin to the tumor of interest. It was designed as non-glycosylated, and I think the glycosylation issue in plants will be talked about later.

Antibody is purified from corn seed used as an injectable therapeutic and compared with glycosylated antibody produced in the mammalian cell.

The protein by physical characterization showed similar quality, and in this comparison we're looking at an aspect of the similar functional quality.

In the figure, in green is the plant-produced antibody; in red the mammalian-produced antibody. And you're looking at the accumulation – the pharmacokinetics in

the blood. Therapy involves delivering the antibody, and over time there's a slight diminution in the blood.

You're really looking for the antibody to accumulate, though, in the tumor, and so you really want to clear out this material that's in the blood by the clearing agent, and you can see, again, a parallel track of behavior since the clearing agent is added to remove any free unbound antibody from the blood.

In order to deliver this type of therapy, not only in a clinical trial but eventually towards commercial and general use, it needs to be a stable genetic system, and that's predictable, again, from the acres, millions of acres, of transgenic products out right now.

This figure shows an antibody, two gene trait. The genes, again, were delivered by a biolistic-type method, two separate gene cassettes, one for the heavy chain; one for the light chain.

It happens that in the Southern pattern here, the upper band is the heavy chain; the lower band is the light chain, two separate gene cassettes delivered.

One of the best expression found showed single-copy-type insertion for each trait. After five generations we harvested three ears out in the field. We picked three seeds from each ear, loaded them up, collected DNA. They show the same sort of Southern pattern.

Further, these are planted and then harvested in the sixth generation. We can PCR out the coding regions for the heavy chain and light chain. That sequence is identical to the sequence found in the plasmid six generations earlier.

Remember, it's not just six generations. There's a lot of cell divisions in making a plant going from seed all the way up to the pollen in the next generation. The genes are stable.

The expression is also stable, as shown in the next slide. This, again, is the seed expression data from the same genetic event that I showed in the last slide. This is a replanting over three generations of the same genetic event in three different locations, three different years.

And you can see in each case the expression given all of those environmental differences would be normal in these three cornfields, fairly uniform expression levels and accumulation of our protein.

That can also be observed in this slide, in one particular year in a large range of environmental

conditions, four different locations. In some cases we split the field here (lots 514 and 515) and here (lots 516 and 517) so that we could look at different harvest techniques, different crop management practices.

Overall with these inbred-type genetic materials, you can see a vast range in the blue bars of field harvest, how much grain you actually get out of the field, six-fold range overall.

But either directly observable in the plant, the crude protein accumulation of the antibody or after Protein A purification, which speaks to the full assembly of the antibody, we only see at most a twofold difference in accumulation.

We can take that a little further in terms of protein quality in this example showing a material grown in '97 in a tropical field versus a Midwest field a little later. We'll grind them up.

The step prior to extraction is to reduce it to a milled powder, store that material for different periods of time under cool conditions, and then process them at the same time.

We have the same yield of antibody. We have the same quality of antibody as seen here by size exclusion chromatography. We can take it further to the point of actual antigen binding in vitro, and we see the same quality, the specific activity of antigen binding.

We need to maintain that trait. And the management of the trait would be similar to what a breeder has been doing over the years with corn and other products. If you remember in our first generation, we have a segregating trait. Only one member of the chromosomal pair will actually have the gene inserted so you'll get a segregation in the first harvested ear.

There will be seeds that will not inherit the trait at all, seeds where there's genetically mixed or heterozygous genetics and seeds that are genetically pure. Although there is a dosage effect we see, these two last aspects are not easy to determine on a single seed level.

Instead, replanting this heterozygous material and this pure-breeding material in this next generation, we can see segregation in the heterozygous seed. These ears are then useful for process development as well as feeding into preclinical and potentially clinical materials.

The pure breeding ears can be bulked, and this would be your breeder seed stock, your pure breeding material that can carry on your trait continuously.

The maintenance of that trait will also follow from traditional breeding and plant management practices. In the simplest method we can continually propagate and maintain and produce from an inbred genetic event.

We'll extend from current breeding practices where typically they'll use an isolation distance to keep this particular genetic event separate from any others at 660 feet, and at that end point they can hit the mark of one off-type seed, a different plant type, different plant color for instance, out of a thousand. We, of course, want to be a little sharper than that, and so we can adjust our isolation methods accordingly.

The bulk of the field can be used for purification of the trait. A subset, depending on your product volume, can be confirmed as breeder stock seed. We'll look for lot uniformity and identity, similar to some of the tests that I just showed you earlier.

Each generation, you can then repeat this process, each generation archiving a portion so that you're able to trace back the quality of the harvest at each generation. That expression of your protein of interest as well as all of the other components in the plant have to be consistent enough to yield a consistent product. We do have some variation in expression level, as you saw earlier. It's not large. There may also be some variation in some of the other components of the plant, variations in starch level, proteins, amino acids. These you'd want to clear out during purification.

Some of the components we test for in the purification process are shown here. Much like traditional systems, specifications can be set, and following that guidance, in this particular case, we were purifying the antibody in a simple three-column process. Seven different lots then can be averaged and compared.

One of the aspects we look at is the size exclusion chromatography purity. In this particular case we'll only be looking at the monomeric type of antibody. Any sort of aggregates that have formed due to what the plant does or the nature of that antibody have been excluded in this measurement.

Endotoxins can be brought in from the plant-produced material. This material is really grown outside; its not a stainless steel fermenter. But we can remove these contaminants to acceptable levels.

Protein A may be carried over from the column matrix. We can reach levels below detection as well as any bioburden that's been carried into the system can be reduced to levels below detection.

The overall protein quality as shown by reduced SDS Polyacrylamide electrophoresis. Shown in these seven lots, a single heavy chain band, a single light chain band, that compared to the mammalian-produced standard, I'd like to note here in the heavy chain there's a slight size shift. This is, again, due to the lack of glycosylation in the plant-derived product whereas the mammalian produced has extra mass added because of the glycosylation.

In summary, then, I think we're working with a system in plants, especially currently with nuclear transformants, of a proven technology that's been shown to work on millions of transgenic acres for agronomic traits.

We can apply those same tools and some others that we need, to yield high expression in corn seed to the point of delivering on a Phase 1 clinical trial for an injectable product.

In that collection of traits, we can see genetic and product stability, both at the level of sequence of the expression cassette, and the level of product to some very strict end points required.

By adapting breeder strategies and incorporating some thinking already developed with more traditional systems, we can reach the GMP end points necessary for injectable products or any other sort of biologics needed and deliver what we see as a unique system, especially for high volume and low cost production.

I don't know if there's time for any questions, but I'd be happy to entertain any.

LOUISE HENDERSON: If you wait just a moment, we have a microphone coming for you, and if you would please state your name for our record before you speak -- and your association.

WALTER GOLDSTEIN: Thank you. Walter Goldstein. I'm associated with Biolex, North Carolina.

I have a question on the antibody that you produced in corn versus that which was produced in mammalian cells. The one in mammalian cells is glycosylated. I remember you said the other one was not.

Did you see any sort of interesting antigenic response that you did in trials? That's my question.

DOUGLAS RUSSELL: Sure. This was -- to get into a little more detail on that collaboration, we were the producers. The inventor of the molecule, NoeRx is still working through the data.

What I can tell you is that their initial data which they're still working through didn't show such a difference. On the other hand, it is a little bit complicated because part of the therapy was using an avidin conjugate, which being a nonhuman protein, was pretty darn antigenic.

So far the verbal that I've gotten back in meeting that they've displayed publicly shows that the antibody performed as desired over the time range that they observed without antigenic issues.

WALTER GOLDSTEIN: Thank you.

YASMIN THANAVALA: I'm Yasmin Thanavala from Roswell Park Cancer Institute. So I guess you don't really have very much more data to share with us on the clinical aspects or the clinical outcome of this antibody.

What were the end points that were being looked for? What kind of patients were these?

DOUGLAS RUSSELL: NoeRx is working to put together the publication. If you get back to me later, I might be able to find you some of their overheads.

YASMIN THANAVALA: Okay.

DOUGLAS RUSSELL: But in general, these were patients that did not succeed in other therapies, so they were fairly sick. It was obviously an acute therapy. There were problems with the design of their trait, and so they're working on how to improve it, their particular therapeutic design.

The data in total, though, showed our material produced from plants without glycosylation performed as well as their traditionally produced.

YASMIN THANAVALA: I'm sorry. What cancer was it?

DOUGLAS RUSSELL: Golly, they hit a number of different types of patients.

Jeff, do you remember?

CRAIG: I'm Jeff Craig. I work with Doug. I think the answer to your question, the clinical trial itself, is the avidin finding, not specifically the antibody itself, regardless --

Your question about the clinical trial itself, I'm not sure. I really don't remember the cancer. It was a cancer antibody that worked for a number of cancers, but it targeted tumor cells.

The clinical trial resulted in the antibody going to the tumor site very effectively, regardless of manufacture of the system, but in this case the clinical trial was stopped because the antibody also bound nonspecifically.

HILARY KOPROWSKI: Can we all put our hands up for who passes the microphone? I have had my hand up for some time.

I would like to ask, one, about the name of the antibody. Do you know what antigen?

DOUGLAS RUSSELL: EPCAM was the epitope.

HILARY KOPROWSKI: Yes. And what did you say? Many cancers or many diseases? Did you say? I may have missed it. Can I have a more clarification about that?

DOUGLAS RUSSELL: I can get you that later. Can I talk to you later about that? I want to make sure I give you the accurate information since it is somebody else's therapeutic.

GORDON MOORE: I'm Gordon Moore from Centocor. One of the advantages of the plant system is the apparent lack of animal viruses and therefore the need for antiviral or viral inactivation steps.

What are the chances that plant viruses may, in fact, interact with human or may interact with animal viruses which then in turn might interact with human?

To what extent can we be certain that we don't have to worry about plant viruses in human therapeutics?

DOUGLAS RUSSELL: The testing of that crossover -- people don't generally look for that data. What you can say is generally you haven't found such diseases in people.

In some cases researchers found an absolute block of the biological mechanisms used by viruses in mammals to be propagated in plants. So in some cases there is real data that says biologically, it won't work. Plants will not propagate mammalian viruses.

The alternative of a plant virus, a TMV, creating mosaic patterns in people, I just have never heard of such a thing.

GORDON MOORE: Would you take the position that it will not be necessary to incorporate purification steps designed to remove virus?

DOUGLAS RUSSELL: I think the column techniques we're using have been proven to remove some of those factors, but it isn't where we target our process validation. We'd be more thinking about some of the factors of the endotoxins that may be -- or protein components that may be particular to plants different from mammalian. We'd put our focus where it's really needed to study.

HUGH MASON: Hugh Mason, Boyce Thompson Institute, Cornell. I just wanted a little clarification on the slide where you showed expression or the recovery of antibody in different field locations or different environmental conditions.

One set of data was showing a greater degree of variability, and the other was showing lower levels, but I think perhaps it was a purified form of the -- yes, this slide. What's the difference in the milligram per kilogram Protein A purified material?

I think you alluded to the fact that that material may be more correctly folded and active, or what is the difference?

DOUGLAS RUSSELL: Oh, I'm sorry. What we're looking at in this slide is the crude antibody accumulated, and in this particular case the way we're measuring that is the assembly by a capture of the antibody by a -- the gamma chain captured and then detection of the antibody with a kappa chain detection system.

In the following slide, though, we've actually purified through the antibody at a 50 gram scale of seed with a Protein A column, s you're reaching a different end point, antibody that can be recognized by Protein A and is assembled to a size -- column exclusion size similar - to a full-size antibody.

HUGH MASON: Thank you.

KEITH WEBBER: I'm Keith Webber. I'm at the Center for Biologics Evaluation and Research, FDA. This question might be directed toward Allen Miller mostly.

You alluded to the use of whole viruses as a vaccine, and I was wondering if there was any information available

on antigenicity of the native proteins that would be components of one of those viruses?

ALLEN MILLER: So plant viruses can be very antigenic on their own in mammals. Is that the question? Yeah. I don't know any data, but I know that people who work with plant viruses had tested for antigenicity themselves and found antibodies, so yeah.

KEITH WEBBER: Maybe there was somebody in the audience as well who --

ALLEN MILLER: Yeah. I'm not really an expert on that.

JIM WHITE: This is Jim White. I work at USDA, by the way, Riverdale.

Allen, comment about the evidence that animal viruses replicate from plants or virus --

ALLEN MILLER: Yeah. I was thinking about that. I'm unaware of any evidence that a plant virus can replicate under any conditions in any kind of mammalian cells.

Can anybody challenge that? I don't think it's been tested a whole lot. I think there's all sorts of barriers. You know, the way plant viruses enter cells, especially animal viruses go by receptors, and plant viruses just get into -- I think there would be a big problem with, you know, avoiding the normal mammalian defenses.

But even at the intracellular level, if you just introduce a cell culture, I don't know of any examples. One of those translation sequences from the virus I work on, my students went on to Harvard Medical School, and there they tried our plant viral one in rats cells, and it didn't work. That's one example.

But there are examples of an insect virus that was shown to be replicated in plant virus.

JIM WHITE: I agree. And I'd just like to say for the people that the food supply is contaminated with plant viruses. The plant virologist, Hector Quemada, is here who worked with Asgrow that surveys for viruses in squashes, and generally, 10 percent of the food supply, depending on where the food is coming from and the disease practice, the viruses can be found, and it's very evident in human diets.

UNIDENTIFIED MALE: You spoke to replication also. Could you extend that reassurance to integration, and also, has any work been done on interaction between plant viruses and animal viruses so that genes or fragments of genes of

plant viruses might end up in animal viruses but could then be in animals?

ALLEN MILLER: Yeah. This is a tough question. I mean you're talking about interactions of plant viral genes with animal? I'm not quite sure what your question is, I guess, but --

UNIDENTIFIED MALE: You spoke to the issue of plant viruses replicating. You were speaking of plant viruses replicating in animal cells.

I was wondering whether there was any information on genomic information from plants integrating into the genome of animal cells, not the virus actually replicating but the integration of genetic material from plant virus in animal cells. That might be one concern.

Second concern might be the interaction of plant viruses with animal viruses, recombination or any other exchange of genetic material between plant viruses and animal viruses.

ALLEN MILLER: Well, at a distant level, I mean, at a very distant evolutionary level when you classify viruses, there are parallels. Like, back in 1984 when sequences were first coming out, it was found Sindbis virus looks more similar to Bromo mosaic virus than Bromo mosaic virus to does to -- I don't know -- some other plant viruses.

There are distant relationships between plant and animal viruses, but as far as evidence of any kind of recent recombination, I'm not aware of any. It's been hard enough. There's just barely some evidence suggesting that any plant viruses can actually integrate into plant DNA.

Pretty much the rule has been plant viruses don't integrate -- even plant DNA viruses don't integrate into -- there's nothing like an integrate retrovirus, although now there's evidence suggesting a banana virus that might actually integrate.

But I don't know of any -- I can't really say much about interactions between plant and animal viruses or -- sounds like a potential way to get NIH funding.

JULIAN MA: For Dr. Russell, I'd like to ask you about aglycosylated antibodies. I'm interested to know if you think this is a technique that would be generally applicable to antibody expression in plants, quite apart from any side effects you might get when you inject them into mammals?

Do you think it might have an impact on quality-control degradation into the plants?

DOUGLAS RUSSELL: In plants as well as traditional systems -- and there's been a lot of data in traditional systems that the glycosylation pattern can be fairly varied. Remove it and there's no variation.

You do affect, though, with some proteins the behavior as a therapeutic. For the purposes I discussed, simply you're trying to drag a toxin towards your site of activity. Other therapeutics also don't need glycosylation because they're simply trying to mask epitopes.

In other cases you really need that glycosylation for the efficacy of the antibody in order to recruit other immune functions. And in that case you really need to define -- have the right glycosylation, have it consistent.

In the range that we've seen in plants, both your own data, published by other labs, what we've seen, it can be within the range seen with mammalian systems. But you can avoid that analytic need just by genetically removing it in some cases.

JULIAN MA: And have you any data on O-link virus as opposed to -- have you any evidence on O-link carbohydrate? Have you done anything on O-linked?

DOUGLAS RUSSELL: We have looked by a mass spectroscopy to see if there is any cryptic addition of O-link because the rules are fairly soft in both mammalian and plant systems. One, we've looked on antibodies in different expression systems. We've not seen O-linked.

With one protein in cell culture, we have seen O-linked, and we could figure out a way around it, but on antibodies and as well in whole corn plant, we've never seen O-linked at all.

JULIAN MA: Thank you.

KEN PLAHN: I'm Ken Plahn from Pfizer. I just want to make a quick comment on the potential for plant viruses infecting mammals.

And I think there was two points for consideration. One would be whether the virus can enter the mammalian cell, and if you look in the trading of vaccinia where you transfer mid-range proteins -- I mean proteins from viruses, say, from cell proteins to viruses, then you may transfer to that plant virus and then enter the mammalian cell.

But the other thing would be that it would have to replicate the cell machinery, so it would have to have the

ability for transcription binding or something like that, which I think would be much less likely.

But it may be worthwhile, you know, as technology looked at that to look at any potential binding human transcription factors to plant genes.

VLDADI YUSILOOV: Vldadi Yusiloov from Biotechnology Foundation and Thomas Jefferson University.

In regard to the replication of a plant viru in mammalian cells, we have tried to replicate the virus, the TMV virus and the CMV virus, in Hhell cells, and we haven't succeeded in anything. We tried to by transmission of the vaccinia virus, and we haven't been able to do it, although we can express perfectly through the vaccinia virus any given protein in the plant viruses, but we couldn't replicate the whole plant virus in cells.

LOUISE HENDERSON: Well, thank you, all. I believe we've reached the time for our break now. I want to encourage all of you to take some time during this break to look at the posters that are to the north of the auditorium and also have a little refreshment.

We are going to start again at 10:30, so our break is a little bit short, but please take advantage of the posters.

(Short recess.)

KEITH WEBBER: I guess if everyone will take their seats, we'll get started in the next session. And before we get started, I have a few announcements to make. One is there appears to be a bit of a pile-up at the telephones, and I want to let everybody know that there are not only phones on this floor here, but there are also two phones available upstairs and two phones available downstairs. So if you are finding that the phones are in use here, feel free to take the stairs up or down to the other phones.

The other thing is that the posters -- we have a lot of good posters, and they're around to the right. You'll find them in the next room, over here to the right. So get your coffee or drinks and head over there and look at the posters.

With regard to questions -- and for this session I think as with the last session, we'll save them to the end, but I think what would be best, if you have questions -- and I'm sure everyone will -- if you could line up at the microphones that are in each stairway here, andwe'll take questions in the order the way people line up.

The second session is going to focus on some of the more pragmatic areas of production and purification of biologics produced in plants and I'm really happy to have two excellent speakers to present in this session.

The first is Carole Cramer who received her Ph.D. in molecular biology and biochemistry from the University of California-Irvine. She's now a professor in plant pathology and physiology at Virginia Tech as well as a cofounder of CropTech Corporation where she serves as the chief scientific officer.

Her presentation will focus on the issues of genetic and protein stability as they relate to biopharmaceuticals produced in transgenic plants.

CAROLE CRAMER: I'd like to thank the organizers for inviting me. And what I would like to do before I get into the issues of both gene stability and protein stability in plants, I would like to sort of put it into context, and I think that one of the issues why you're all here and feeling very curious is the concept that when you think of biologics and the current way that is now emerged as sort of the traditional bioproduction system, you think about one of these. At CropTech we call these GBRTs, great big round things. And GBRTs are now the way you're used to.

And what is the concept that emerges. First of all, you've got, you know, white-suited technicians with the little booties on, and the concept is complete control of the environment, is high levels of sterility.

What are we talking about here? All of a sudden, we're saying we're making the production stream initiate potentially in a field or greenhouse.

What are the images here? Well, first of all, you can barely see it, but under there is dirt. Now, in agronomy we call this soil, but this is dirt. Okay? You've got bugs crawling on it. There might be snakes running around here. And you certainly can't control the heat and the light because it's coming from the sun.

So all of sudden now, you're in a hugely uncontrolled environment. And the real task is to say that this environment is as safe and reproducible source of pharmaceuticals as this environment.

Well, I'd like to take you one step further. Here is a little picture of the rain forest, and there is many drugs currently on the market that are actually sourced from wild species that are collected and extracted.

And in this situation, first of all, not only do we not control the environment, but we know nothing about the genetics. We don't know whether the same tree if it's harvested one year from the other is giving the same level of bioactive material, and yet this is currently an accepted source for many pharmaceutical drugs, small molecules that are on the market.

Well, I would like to say that, in fact, a field of tobacco or tomato is a lot more like a GBRT than it is like a rain forest. What large-scale agriculture has selected for is extreme uniformity. And in fact, the constraints, the cost constraints and the reproducibility constraints, in large agriculture is extremely stringent.

We currently have, I think, currently in the order of 20 million acres of herbicide-resistant soybean is grown. The transgene that is entered into the soybean allows you to use a certain herbicide. If 1 percent of those seeds actually have lost the transgene or no longer showing resistance, this is a huge loss from the agriculture point of view. And so the tolerances in the agriculture field for variation and for instability are very small.

And so these are the situations that this is the biomass material we're starting from. And what I would like to do is show you that this is actually a very stable source, genetically very stable, and then to talk about two different systems that have sort of emerged that are very opposite as far as your ability to not just produce in the field with uniformity but then look at extraction and show uniformity in that situation as well.

So the two systems I use is basically I've gotten some data, very nicely provided to me by both ProdiGene and Monsanto's IPT group looking at the production of transgenic corn seeds, much of what Doug Russell just talked about.

And then I'll compare that with CropTech system where we actually use the leaf material of tobacco so it's a fresh green leaf as our starting material, but we do a situation that's quite different. It's what's we term post-harvest so that we actually don't express the human protein in the field. We utilize a wound-inducable promotor so we actually can harvest. We can store the tissue for periods of time.

We then turn on the transgene by literally running it through the equivalent of a paper shredder on a very large scale. And so this actually activates locally a wound response in the human gene is then produced over the next 24 hours so that we effectively separate biomass production from our actual pharmaceutical protein production. And this happens in a very discreet and small period of time.

So what really are the stability issues? Our first question is, is the transgene stable from generation to generation? And in the practicality of how we do our business, we tend to select for two things in a line that we're going to move forward with.

One is that you actually have to phenotype the expression level and the protein product that you want. The second is that we tend to go for low copy number, and I'll give you an example of why we do that in a minute. But that facilitates the downstream genetics. It facilitates uniformity, and it also minimizes this issue of gene silencing that was talked about in an earlier talk.

Unlike animals, once we've gone through a seed, there's no such thing as chimeras, so our transgene is expressed in every single cell of the plant. Once it's there, it functions with regular mendelian genetics, and it shows the same very stable phenotype and retention that you would see with any native plant gene. So we don't see that bacterial or human genes put into the genome have any problem with looking just like a gene.

Doug Russell showed this gel in his work, and I just wanted to reiterate that it has been demonstrated that once you get this gene in, you have incredible stability. And so this is now your sixth generation, and this is looking at individual ears from different kernels or from different years -- individual kernels from different years in a field. And basically, changes. So it's very stable in the genome.

There are issues -- I mean what you would like is a very consistent system. So you're looking at plant-to-plant variation, which can be impacted by copy number and segregation. You're looking at generation-to-generation variation and trying to define your master seed stock.

And finally, this issue of environment, and what are the site-to-site variations? How does that impact the quality of your product, and also, how does it impact your ability to express your transgene? So I'll go through a number of these points.

Here are some data that is looking at the first 40 transgenic plants that we pulled out that were expressing a human urokinase gene. And it looks a lot like Doug Russell's data. You basically get a range of gene expression.

This is what we term position effects in plants, and you can take the same transcript or the same gene, and depending upon where in the chromosome it lands, you get different levels of expression.

But essentially, if you look at your highest expressors, which are the ones that you want, you tend to have one to three copies of the transgene. We generally go for the single copy. But if you do the same genetic analysis with your lowest expressors, they can have one to three copies.

So the point is in plants it appears not to be so much the copy number that enhances your expression but the particular site of integration.

Once you have that gene, however, then it responds and acts like a typical mendelian gene. So this happens to be plants that are expressing human serum albumin, and if you go through your generations and look at the homozygous so it's now a single gene but it's now in homozygous form, so it has a single copy, or the progeny, what you put in homozygous form, essentially, you get the expected doubling of production based on going from a hemi to a homozygous.

So with tobacco we have some advantages for moving to a seed lot that we consider a master seed very quickly. And this includes the fact that we can start with elite lines. So the initial plant that we're transforming is actually the plant that's going to the field. And that's because these lines are very easily transformable.

Tobacco naturally self-pollinates, and a single tobacco plant can make up to a million seeds, so it produces a beautiful flowering head. Each of these flowers becomes a seed pot, and literally, you get millions of seeds smaller than a poppy seed.

What that means is that you can scale to a huge seed bank very quickly. And essentially, you can have unlimited homozygous identical in uniform seeds generally in two generations. If you've got segregating genes so you've got two copies, say, that may take another generation.

This turns out not to be the case in corn, and I think that Doug Russell sort of made it sound a little bit easier than it is, but in corn you tend to have to introduce your gene into what's called an embryogenic line, so not all corn regenerates and transforms as efficiently.

And so what you generally do is you introduce it into an embryogenic line, and then to move to the field, you do a series of back-crosses into your elite lines. And so this is data that was provided by ProdiGene where it was some of their early work where they introduced Avidin into transgenic corn.

And they use the gene gun, so they started with a lot more copies in their initial transgenic plants. And so what

this represents is a series of back-crosses where they take their transgenic and isolate a good expresser. They cross it to an elite line. They then go the next generation, find a good expresser, cross it back to the elite line.

So what you see and why you see an increase over these generations is two things. One is that you're selecting for one or a few very high-expressing copies that you're selecting each time. But more importantly, you're now crossing it back to an elite line. It gives you a much better quality seed that has higher level of protein in the seed.

But once you've gotten to this point, you actually stabilize very -- it becomes very stable. So once you've selected and got your elite line characters back, then this is now Monsanto's data. You basically get a very stable and reproducible system.

And so this is the same data that Doug Russell showed. Basically, if you look at a series of different plantings of one line, so you now have two different master seed lines that have been developed, even though you have significant differences in yield from field to field showing the variation of the impact on the growth of the corn, if you look at all of those, essentially the level of expression is very consistent.

And as Doug showed, not only is the level of expression per kernel of corn very consistent, but if you actually do the mass spec on the purified monoclonal, you can see that the product is extremely consistent.

When we look at upstream and downstream using transgenic plants, I think that this concept has to be broadened to some degree. And so we talk a lot about the biomass production but how this session will move is taking it not only from a how are you going to grow this stuff in the field but how you're going actually harvest it, how you transport to facilities, how you initiate extraction and concentration, and eventually how you will then move into a normal downstream process.

My contention, and I think sort of the general experience, is that once you get into a bulk extract, the purification downstream is very similar to what you would do out of human serum, out of cho lines.

Where you really see some differences is going to be in the upstream, and those are some of the issues I would like to look at. This protein stability in -- as all of you know is really protein dependent, and so some proteins are very stable; some proteins are very unstable. And these

things we can't change. And if they're unstable in human cells, they're likely to have similar issues in plant cells.

But there are things that you can do to impact the stability of your protein in a plant cell. And these include the site of accumulation. Basically, you would like to move them away from compartments where there's lots of proteinases.

It involves the length of time that I call in planta storage, so that can either be in a flesh-weight leaf or the amount of time it would stay in a desiccated seed. And then there will be issues such as glycosylation or polymerization that will impact stability.

So first I'll talk about seeds. Seeds are really remarkable. Seeds are nature's system for long-term high-value protein storage. What a seed does is takes all of the nutrients and proteins that that plant is going to need for germination the following season and puts it in a package that will allow it to go stay in the ground through freezing, will allow it to go up to 90 degrees or 100 degrees Fahrenheit in the field and still maintain complete viability.

So you actually have excellent storage of very high levels and exceptional shelf life and an interesting thermostability.

So this is again data from ProdiGene where they're looking at they have transgenic corn now, lines that are well developed, and they're looking at the stability of a reporter gene, GUS, in various storage conditions.

And these are fairly short times here. We're looking at just 100 days. In other papers where they have Avidin, they now show that the Avidin levels and quality are reproducible well over a year in a corn seed.

And so this is just comparing storage and not just ground corn, so it's actually not an intact corn kernel. And if you store it frozen, you have very stable expression. If you store that at 10 degrees Celsius, it becomes just as stable.

And interestingly enough, if you store it at room temperature, it's also very stable. They have a stored 37 degrees, and I think if you have fully desiccated seeds, this probably wouldn't be an issue, but what Joe GOLPA has suggested is that this particular experiment, they actually had -- at 37 degrees they had fungal contamination, so they threw the seeds away.

But the point is that this is very stable. Just anecdotally, somebody came to me the other day, and they wanted some GUS seeds for experiment. So I had some tobacco, some 35S GUS tobacco seeds. This had been sitting in a little envelope on my lab bench, you know, when I used to do experiments, and from 1991 they were harvested. And first of all, I didn't know if the seeds were still viable, so I threw them on the filter paper, and everything grew up. And then I just took some of the dried seeds, and I ground them up and threw them in an Exclude and put them in the incubator. And those things were blazing.

So literally, those seeds had been sitting with no care in the back of a lab bench in a little paper envelope for nine years and showed incredible levels of protein that had been stabley expressed and maintained in stable form for nine years.

One of the issues when we're talking about vaccines in corn, for example, that would be delivered to livestock is that you often have steps in the milling process, and in some cases this involves high temperature.

So ProdiGene has also done a number of studies to look at how a transgene survives in a seed or during temperatures that would be analogous to what you might see in the milling process.

And so this is looking now at hours of -- so again, you're looking at your GUS enzyme activity, so the activity of an enzyme. And you can see that over 8 hours at 50 degrees, there's no diminution of activity.

You start to see at 70 degrees some decrease, but if you look at period, say, one hour, you still have something in the order of 90 to 95 percent of your activity.

And this probably is analogous to the types of temperatures that you might have during a milling process, and it suggests that that's not going to take you out of business, that, in fact, you have the type of stability in a kernel that will allow you to do experiments.

As you know, the higher temperatures, 90 degrees and 125 degrees Celsius, you can see that, in fact, as you would expect, you get denaturation of the protein.

The good news is that there are already many, many systems since protein stability and protein quality is something that feed corn has looked at for literally, you know, decades, there are systems that will allow you to stabilize this temperature and go for production of recombinant proteins.

Well, our system in tobacco is quite radically different from the idea of a stable seed stored, and I'll just talk you through the process a little bit.

We actually grow in the field, and we need a high-quality leaf. We need it to be young so that you can extract it easily. You then transport it to a bioproduction system.

Turns out that you have a lot of flexibility in storage here. Nobody likes to think about it, but the head of lettuce that's sitting in your cooler at home is fully alive and metabolically active, and when you cut it up for a salad, you're actually triggering a whole pile of gene activation events, and it starts making new protein. You don't think about that from the animal side. Plants are alive and metabolically active.

So we can actually take this and store at room temperatures for days. We can store it in a cold room for up to six weeks and at that stage actually induce it so we run it through a slicing machine. You end up with very thinly sliced each. The wound response is a local response so that here's actually wounded leaf strips from here that have been stained with Exclude for GUS activity, and you can see that you get a very dark strip of activity right at the edge. But we've optimized it for getting every one of those cells to actually express, and stem cells will express just as well.

And so the idea here is that you can store your material if you need to. You induce it. Over the next 24 hours, all those leaves do is crank out human protein, and you can then collect it.

Everything that you're looking at has been made between eight and twenty-four hours before extraction so that you have all fresh protein. And then you can recover it, either from a secreted fraction or in some cases grinding up the leaf.

So we now have issues of not how stable is the corn seed, which has been proven to be very stable, but how stable or how robust is our expression system, our cost, and different harvest conditions?

So here's some data that was generated in a field in Virginia last summer where we're looking -- we grow tobacco very weirdly, so we actually grow it a lot more like alfalfa. We grow it up to maybe 3 feet in height, and then we actually go and mow, and we take that back to the processing facility.

In the next four weeks, tobacco will regrow back up to 3 feet. We go, and we mow it a second time. We come back four weeks later. It's back up. We mow it a third time.

So this is actually from a single set of field plots, but it's mowed essentially four weeks apart. And what you see is that initially our tobacco -- so this is protein yield. The total protein yield is very consistent.

But by the final harvest, our plants are actually getting a bit more lignified, and we have a reduction in our yield of protein. But if you look at GUS activity, so our actual transgene, the ability to induce transgene product formation, once you've taken them back to a processing facility, it stays very stable.

And in fact, this GUS is expressed as GUS per milligram soluble protein, and so if you look at the actual yield of GUS from the per fresh weight, it actually stays constant through the entire season. So there is some level of stability.

What we've also done, sort of the, you know, if you're a molecular biologist, a gene doctor like me, very interesting experiments, which is just looking at what do you have to do to these plants to make sure that you've got expression?

And we've found that you can actually change harvest times. You can change harvest heights, and the system remains robust. But there's also some very interesting practical implications.

We've looked at whether you just harvest the plant and it sits in the field at 90 degrees in southwest Virginia for four hours after you've cut, and then if you harvest it and induce it, do you still get activity?

And it turns out that these conditions have no impact on your ability to induce your gene in subsequent processing. Whether it's left sitting in the field for a couple of hours, you can store it at room temperature for one to four days with no impact.

And interestingly enough, you can also store it in the cold for up to six weeks with no reduction in the ability to subsequently induce gene expression. So that gives us some flexibility in the processing of this material.

However, we have learned -- I mean all of you guys that sit and pile your grass clippings in a pile realize that over a couple of days, you get a little composting, and so among the things that we're testing are ways to store and move material so that you effectively have air circulation

such that you don't get essentially compacting of material before it makes it to the processing plant.

And this actually isn't too hard to do as long as you keep them compact tobacco plants because of the stem structures allows you good air movement.

Well, I mentioned that the site where you accumulate the protein can also impact the expression levels, the amount of product, and the quality of product that you get. And so this is actually a picture of a plant cell. If you look at an animal cell, the big thing in the middle is a nucleus. If you look at a plant cell, the big thing in the middle is a vacuole, which is the equivalent to a lysome. It's organelle. And this thing is packed full of proteinases.

So what you want to do is sort of avoid this guy if you can do it by any chance. So places that people have accumulated transgenic protein are the chloroplasts, the plastids that Doug Russell talked about. And you can actually get very high levels expressed there.

People have expressed it in the cytosol, and that tends to be a less optimal organelle that clearly does have proteinases activity, and I'll show you some data on that in a minute.

People can express or you can put on ER retention signals, and in many cases you can actually increase the level of a transgene product by doing this.

In some cases you're not going to be able to use it as a therapeutic if you've got an aberrant signal. So there are different applications for that.

But interestingly, if you take a protein that is secreted in humans and you introduce it into corn or you introduce into tobacco, it actually is effectively secreted out, and it sits in the APOPLASTIC space between cells, and as tissue cell matures, you actually have regions that have relatively large APOPLASTIC spaces.

So it turns out in many cases, you can get very high level of accumulation of human proteins that are spit out into the cell, and this seems to be a relatively proteinases poor environment that allows you to accumulate proteins.

And this is just some data showing that. These are when we made our first urokinase constructs, we put them both plus and minus the human signal pepcide.

And so what's showing, this is the same slide I showed previously that is actually urokinase that is collected from the secreted fraction. So this is spit out of the cell.

And we have this nice range of activity, and we now are working with these very high expressives.

Just to give you a level of comparison, human urokinase is currently expressed or is purified from human urine for Europe. And the level of activity in human urine is right about the equivalent of those first three little blips right there.

And so what we're seeing secreted in tobacco extracts is a much higher initial feed stock concentration than you would get out of human urine.

If you now compare what we got when we eliminated the signal peptide and so now these are being produced again -- it's just the first 40 random plants that we looked at -- you again have a range of expression, but look at the scales here. We essentially are at about 10 fluorescent units here, which this first line right here is 500.

So by shipping it and storing it in the cytosol, essentially, we got next to no activity. There's two things -- well, there's probably more than two things but two obvious things that this could reflect.

One is the fact that there may be a lot more proteinases in the cytosol than there are in the extra cellular space. The second is that this form of urokinase is not glycosylated for urokinase activity in serum, but they may have something to do with the stability.

And so it's not clear whether this is just the cytosol is bad or that having it in a non-glycosylated form makes it more vulnerable. But there is now a lot of data from a number of different places.

If you look at the Monsanto and ProdiGene with their major products that are moving forward, in all cases those are secreted proteins that move through the endoplastic verticulum and were deposited in the avioplast.

So we have a situation in tobacco where we have cheated one more step, and we've taken advantage of our shredding, which is when we actually take the shredded material, it's now been making human proteins for 24 hours, and we actually just expose it to a buffer solution. We actually can extract about 95 percent of a good secreted protein into that solution in a matter of minutes, which means that we don't ever have to grind up the leaf.

So this is actually a little hard to see, but up here you've got our shredded tobacco leaves. And in some cases if you've got an intracellular protein or a membrane associated, you have to grind this leaf up, and then your task is really to pull your human protein out of this green muck where there's probably 30,000 tobacco proteins that you've got to look at.

In contrast, we found that we can take the secreted -- this is actually secreted fraction concentrated 15 times. This is obviously a very optimistic cartoon, but in reality, what you're looking at is something in the order of 100 to 300 tobacco proteins that you now have to secrete it with.

But we see this as an advantage not only in giving a very rapid way to do your first purification step, but it turns out that you've also eliminated the release of a lot of those other cells that are containing that big pocket of vacuole proteinases.

And so one of the things that we found in this system is that it tends to be quite good as far as the lack of degradation of human proteins.

So what I hope I've shared is the fact that you can take very radically different approaches to producing recombinant proteins in plants, and in all of these systems, the concept of genetic stability seems to be very well documented.

There are different issues, and one has to be very careful in documenting the fact that your product is stable not only -- or your expression system is stable not only in the field but also through harvest, storage, and initial processing.

But we feel confident that because of the advantages of transgenic plants for safety issues, for cost issues, and for the ability to actually meet very large markets that, in fact, these will be seen as acceptable vehicles for the manufacture of biopharmaceuticals.

But it will be contingent on the fact that we do an excellent job characterizing the transgene, the transgenic plant seed stock, that we control the consistent manufacturing process with well-established SOPs, we demonstrate both the genetic and product stability, and that we establish criteria for the specifications of lots and lot release criteria to make it a real pharmaceutical industry.

And with that I'll stop and thank you very much.

KEITH WEBBER: For the second speaker I'm pleased to present Dr. Barry Holtz. Barry received his Ph.D. in

biochemistry from Penn State University, and in the early '80s he founded a bioprocess development company which eventually merged into what is now Large Scale Biology Corporation, which is a company that specializes in the manufacture of biopharmaceuticals in tobacco.

He's currently the vice president of biopharmaceutical process development and manufacturing, and his presentation today will focus on harvest methods and purification using that system.

BARRY HOLTZ: Thanks a lot, Keith, and thank you all for inviting us to present. Keith has asked me to focus on some of the issues surrounding the regulatory strategy of manufacturing, and while we can discuss an enormous amount of stuff here on these different systems and producing things, what I would like to explain to you is using our manufacturing facility to go through a real case study of how we have scaled this up and what are some of the manufacturing regulatory issues that we face.

I think one thing that's important to say is that you can go to the big book of fermentation, and you can go to the real golden book of CHO cell manufacturing, and you can figure out through case studies, models, and products that are very vigorous and robust in the business how to do these things.

In the case of recovering biopharmaceuticals from plants from recombinant plants, the big book is being written, and we've had the pleasure of doing this over a period of about 12 years.

It's also one thing to have a spot on a band on a gel and, you know, hundred milliliter flask full of materials. It's yet again another thing to produce kilos of pharmaceutical-grade protein from thousands and thousands of kilos of >plant biomass.

So I'd like to say that our name used to be BioSource Technologies, and it's very difficult for me to switch over to Large Scale Biology. We've had to do that because there are other companies that have very similar names, and we bought a company that was named Large Scale Biology.

So when you combine the idea of the fact that we discover 400 genes a day in our genomics operation, we can analyze 5 million proteins a week in our proteomics facility and we can grind 6,000 pounds of tobacco an hour, that probably qualifies us for this name. Also, we didn't have to pay a lot of lawyers to figure it out, and that's always a treat.

The development of this time line really has two -- it's biophasic, and it's perfect that we're here today with both USDA-APHIS and FDA because both of these agencies are involved in our production of proteins.

And this really starts back in 1991 with our field release of really the first recombinant virus in the field, which was one of our tobacco mosaic virus constructs.

And I put this time line down not to go through it in detail but to tell you that this takes a long time. This is not something that you will glibly put in your IPO saying that you're going to contract manufacturer or other body of people are going to do this for you in short periods of time. This takes a while to do.

This has been kind of trod over several times, and I appreciate Guy and Dr. Miller's efforts this morning to prestage this so I can go through this really fast.

The comparison is very straightforward. In the case of the transformed plant, we really do transform the DNA. We put a permanent trait or permanent gene into the plant, then use the plant translation at mechanism to make proteins.

In the viral vector business, our vector RNA is infected into the plant as far as in a transfected vector, and then we use the replicate of ability of the virus to make more protein and harness the cellular machinery to the plant to make our protein of question.

Tobacco mosaic virus is a good candidate for this, and that's been gone over. Just a few of the things that are important other than the molecular biological things that were very well described earlier.

The virus has to spread rapidly and systemically to get high yields of proteins in the plant. That's a very good trait of TMVs. It's also not seed or pollen transmitted and not vectored by insects, which gives us very good containment. In fact, you'll see later that we have to do some very robust things just to make a plant infected.

It's probably one of the most well-known viruses on the planet. Virtually every amino acid has been mapped. This is a mutual X-ray rendering of the viral coat, so it's probably the most well-known virus on the planet.

The genome is very simple. It's talked about earlier. What we do is insert a new gene driven behind subgenomic promotors into the genome past the movement protein which is our 30K protein. This protein allows the virus to go from cell to cell through the plasma dismoda and behind the

protein promotor, which as you well know is a very high producer of protein.

So our foreign gene is put into this set. Expression is rather dramatic. Here are plants two, four, and six days post-inoculation. In this case we've used green fluorescent protein tied to the virus as a marker for expression.

Under UV light you can see the local lesions on the second day that are formed by the virus after it's impacted onto the leaf. These local lesions then hit the vasculature and start to move. And by six days post-inoculation, you have a complete systemic movement of the plant. We call this our field of green.

It also infects the entire plant, demonstrated here by looking at the roots that is in this particular photograph.

For field production using viral vectors, the transcript is made into a naked RNA, an infectious RNA. We package up the RNA after it's been modified under the gene of choice in the greenhouse ROME DETOCISA species that we use for packaging host.

That makes our inoculant. The inoculant is then taken to the field and sprayed on plants, and the plants are recovered by harvesting after the vector has done its job. That's typically between two and three weeks after inoculation.

All of this has led to our new bioprocessing facility in Owensboro, Kentucky. We obviously need to be near tobacco to do this, so we're very pleased to be in the Owensboro area, which is the heart of the belt in Kentucky.

This is a rather robust facility. Again, we do render biology on a very large scale. It's 6,000 pounds an hour. There are two unit processes or two divergent forks to our process flow chart, and the first is the preparation of construct and the preparation of inoculant.

At the same time we are growing transplants in traditional tobacco growing -- these are virus-free transplants -- and doing field production.

They converge during inoculation. We then produce the virus in planta and consequently produce our new protein, harvest. We extract, purify, and then package. And we'll kind of go through these steps in the reality of it all and show you how we do it.

Inoculant preparation is done in a very controlled greenhouse situation. There are laboratories in California. These are kind of huge. We actually have our own center as

we plant a different spacing than most traditional tobacco growers.

Each transplant is individually planted on spacings that we've determined to be optimum for biomass growth. If any of you have grown tobacco before, you see the trade at which those plants are being distributed. You'll know that's not your typical burley field in Kentucky.

Then we have to have our head gene jockey come out and preside over the inoculation. This is Larry Grill. Without that nothing works, so we've insured him for enormous amounts of money.

The inoculant is simply diluted in a buffer with a little bit of grit. Then we literally have to go out and sand blast the leaves to get infection. You have to wound the plant in order to infect it, and depending on how fast my colleague is driving the Honda four-wheeler, we can inoculant about five acres in an hour and fifteen minutes. And of course, no inoculation day is on any less than 104 degrees at 98 percent humidity. It's one of Murphy's mandatory things.

And the harvest system, the first time a tobacco grower sees a tip of our cutter go through a field of standing tobacco, his eyes are about this big. Then the second thought in his mind is I don't have to go out there with a knife and harvest these individually, hang them, cure them, strip them, and this guy is paying me to grow this crop, so this is a good thing.

So there's very much of a row crop mentality in our growers, and our growers are very much our partners in developing a lot of these agronomic techniques.

We harvest into these special wagons. We build these ourselves for not only containment but for high-speed delivery to our factory, which is -- we grow within about a 45-minute radius of our place.

The materials, the wagons are – you can't see this little screen. Well, anyway, the wagons are sanitized by spraying with bleach in the field and covered and transported to market, to our plant. We transport about three tons at a time. We have a fleet of these wagons, and each one optimum carries about six tons.

The strategy for recovery is very straightforward. We have to rapidly separate out the biggest single contaminant which is RuBisCO, the F1 coupling factor protein. We accomplish that in about the first 19 seconds after homogenization.

We want to go to earlier barrier separations where we can bracket the molecular weight of the material and also reduce the volume. Reduction of volume is very important in the cost of the process because we want to move very small volumes forward where we have to use expensive reagents. The point of at least 100 to 150-fold reduction, we use no exogenous chemicals on the materials. It's all mechanical processing.

What this also does is really provide very nice waste minimization program because we don't have to resuspend, resuspend, resuspend like you would at CHO and E. coli systems with expensive buffers which are a large part of the cost of good soil.

Typical CHO and E. coli systems, you'll see numbers upwards of 30 to 35 percent of cost to goods are waste treatment cost, and that cost is not going down. And this system where we have a linear reduction of volume, you're looking at 6 to 7 percent. The other nice thing is that our waste products, most all of our solid waste products go right to the field from whence they came and are plowed in as spread manure.

Again, I said we did biology at a very large scale. This is our disintegrator. It's a 76 horsepower RACE disintegrator and has the ability of very little strain of gobbling up 6,000 pounds of leaves an hour, which is a typical rate.

We go through robust clarification, some upstream manipulations of the material. I'll talk more about how we control these processes later on. This is the inside of our processing bay.

We get very rapidly to ultra-filtration, and it is a key part of our technology that we are able to ultra-filter these flow streams at very high rates and capture our products of choice.

The rate at which we make extract, you might be interested in, causes a 20 gallon a minute flow of green extract through the building. So this is indeed large scale.

Downstream purification, as Carole said earlier, we face the same issues in chromatography and purification that other people using biological systems for production.

We have our own QA facility, of course, on the plant. We also have a GMP pilot plant within the building which is a totally environmentally separated area so that we can run two products at one time and develop our portfolio of processes and product.

These are examples of some of the materials that we've made. We weighed over 200 different proteins over the decade or so that we've been around. These represent some of the diversity of compounds that have been made.

The two top ones are ones that are going forward into the clinic at this particular time. One is Galactosidase, which I'll use as an example today. It's human alpha-gal for treatment of the Fabry disease, which is a disease that is a genetic-inherited disease where the gene coding for this particular enzyme is not present.

We also are going forward with a patient-specific non-Hodgkins lymphoma vaccine. And that's in collaboration with Stanford. We've done a lot of these things before. We're not new to large scale businesses, and I just put this slide up to say that we've come from the fermentation business. We do know what a GBRT is.

We've also been in the multiple enzyme natural food flavor business. That business is still -- we sold it to Nabisco. They still operate that plant. That plant grinds 4,500 pounds of plant 16 hours a day 115 days a year and distills off all of the flavor chemicals at 6,000 gallons an hour, so we have a lot of GBRT experience, if you will.

We do do concurrent bioprocess development. My colleague Larry Grill is here. He's in the gene jockey side, and I'm on the pots and pans side. We certainly work in parallel from the beginning.

As soon as the construct hits the house, we are worried about concurrent development of bioprocesses as we optimize in the molecular biology.

Our process development group is kind of interesting in that we have bundled bioprocess development. The protein analytical and separations groups and the agronomics groups all fall under the bioprocessing, and that gives us a very integrated team. You'll find out later on that all of these folks are absolutely mandatory to pull off the GMP facility.

We start this journey by acquiring a facility from research efforts which is part of Swedish tobacco. They had a little junk work extraction facility in Owensboro, and that's what got us there originally.

The important part of this slide is that we built this pilot plant full scale out of old parts and pieces and PVC pipes and used tanks and everything else to prove the scalability of the process. And you'll see, again, why that paid off very, very hugely.

And that's exactly this concept, is the use of a scaled-down approach to validate your process. Large Scale is indeed its own set of circumstances, and we have used the development of the process in Large Scale to validate our pilot plan and bench-scale activity. So we've kind of done it in reverse. But you also then know what the unit processes are and how they're going to behave and how much they cost.

It also really minimizes your risk in scale-up capital. You can start prevalidation. You can start all of the pro forma aspects of your process. You can also spend a lot more time designing the GMP facility rather than worrying about whether all the parts and pieces are going to work when you scale it up at the same time. So a lot of the pharmaceutical regulatory issues are able to be focused on once you can define the process in full scale and don't have to do the two in tongue.

We also had to reinvent the book on tobacco growing. The agronomy of leaf tobacco for smoking is not the ideal for growing large amounts of virus and proteins in plants. We spent a decade or so worrying about that. We also have had to design and build our own farm machinery, as you've seen before.

Now, how did we do this? We went from concept to operation in two years, which we think is pretty good. One of the things we were not encumbered by was a consulting engineering group like a BOARD DANIEL or somebody -- apologize if somebody else is out there -- who charges you your business and then feed it back to you with a lot of paper and dead trees. We had no fear about putting two pipes together and turning the valve on to see what happens.

So our design team was the project engineering group, which was from our group, a contractor, architect, and the regulatory team. The execution team was very much the same group, and that was the thing that really made it work in that Steve, my colleague out in the audience, and I lived in Owensboro for probably two years 80 percent of our time, and we were able to make decisions in real-time on the development of the site.

So we built it in June of '97. We turned it on in July of '98. The team had to be coordinated. Software coordination was mandatory, both for validation purposes and to keep things going. We all had to be on the same software page. Bill Gates won that battle too.

Now we'll talk about the regulatory aspects of this thing. I think being in a new business, a lot of us don't know very much about GMP, especially when we were coming

from much of an R&D environment into a manufacturing environment.

Steve and I like to say when we go over the continental divide, we put our manufacturing hats on and take our R&D hats off as we're soaring along the United Airlines at 32,000 feet.

But GMP is something you do, not something you have. Just because you've got a lot of binders in a room and you've got all kinds of regulators running around and clipboards and all that, it's a very dynamic process, and there are two basic questions you have to answer at the end of the day, and I think this summarizes it.

If the inspector is here, it's very fair for him to ask, "Can you deductively prove to me that you can make this molecule the same way, efficacy, potency, purity, and safety every day?" And the second question is, "Would you be happy injecting this into your 12-year-old son's arm?" And I better have very good answers for both of those.

So this is the deductive battle that we face, and this really means that the process development has to be very robust, and documentation has to be excellent.

So construction was no less of an issue, documenting it. One of the things, we went through enormous amounts of documentation – I won't bore you with every piece of that, but one nice thing is we digitally photographed every pipe and piece behind a wall or under a slab and numbered it and also tagged it.

So when our inspectors come from the agency and want to know where this drain goes, what it's connected to, we can visually demonstrate that very rapidly.

We also have a long-term site plan. Right now our site consists of one building in the upper right-hand corner in this large greenhouse complex and the lower corner, but we're also planning ahead for seven different buildings on the site. It's a twenty-one-acre site.

Our thought is that one of these buildings will manufacture one drug. We don't want to get into multiple drug issues in the building in large scale manufacturing.

The fortune part for us is that we do not have, as Carole affectionately referred to, is the GBRT issue, which is about 80 percent of the capital in most traditional drug manufacturers. So we can afford to build multiple buildings, and if we do it with a good plan and good centralized utilities and so forth, it will work out well.

We started our facilities validation master plan in 1997 when we were running the pilot plan so we could start to prevalidate our processes.

Plants do present a new regulatory data base that needs to be generated. However, cGMP is cGMP, and I'll say that more than once today. We don't see anything plus or minus that's going to be anything -- there will be no paradigm shifts at FDA. There won't be any changes in the way GMP is done, and it's a very blue-collar effort. You have to do the work.

Plants present unique challenges, just like CHO cells and E. coli. There's not going to be any shortcuts, and we don't think that there is.

We have a lot of manufacturing control issues. Some will need to be validated, and some won't. The basic tenet is if the unit process that you can control affects the quality of the product, you will have to validate that unit process. It's as simple and as complicated as that.

So we look at all of these sorts of issues over the broadest part from the agronomics all the way to incoming raw materials for our total validation effort.

We have used a turnover package approach to prevalidate our facility. This is a system that provides an integrated history of all of the unit processes of your facility. This is pioneered by some friends of ours at Banzinger Banks.

It's a very systems approach to documentation where each system has its own life cycle and its own history from the preengineering through the engineering specification design, installation qualification, operational qualification of all of these subsystems. And it also provides -- I hate the cliche but a living document of all of the system development that you've done for each unit process.

It also makes change control very rational and very clear. And it makes it very clear to the inspectors when they want to deal with issues of change control. You can rationally show how these things were engineered and developed in one binder for one system, and it's all in one location.

It sort of works like this. Again, I've got to give credit to Banzinger Banks. They've helped us out a lot in this and are really compliant with this particular thing. But project team starts from the beginning and follows the

unit process development and validation all the way through until we get an accepted system.

The system is turned over in its final form. Your P&IDs are locked. Your SOPs are locked. Protocols are locked. Systems are validated and then are turned over to the manufacturing. And then if there has to be a change order or change control procedure put in place, it's very easy to document and decide whether revalidation is necessary or not.

Again, raw materials definition and handling, we have to audit vendors. All of these things -- and some of you know this very well, but some of you don't, so we'll go through some of these things.

But we have to go out and audit virtually all of our key vendors to make sure that those products are made properly. We have to receive the materials, quarantine the materials, QA the materials, and distribute the materials with complete chain of custody documentation.

We have unidirectional process flow. The only reason I put this floor plan up is we start in the upper right-hand corner, and the product proceeds to the upper left-hand corner down to the lower right-hand corner and out the lower right-hand corner of the building. So we always have a unidirectional product flow during the process. This is another key regulatory issue.

Electronic batch records. We had to write our own software for this thing, and we also had to write our own software challenges to prove that every digital signal is recorded in a batch record, fire-walled sufficiently, and protected from any contamination in here externally. The easy way to do it externally is to pull the plug out of the wall so it's not connected to the real world, and that helps a bunch because, again, Bill Gates has made everything so porous for us that Windows NT is the only way to segregate it or to keep it safe to segregate it.

We have our upstream is highly automated, and there are over 240 IO points in the building, and all of those are a part of the batch record. We've developed electronic formats and a secure data base to handle that situation.

Now our regulatory activities with APHIS. This represents a second phase of regulatory issues, and how do we handle the agricultural side? We started back in 1991 in very small plots and have worked diligently, fortunately with the same person. Jim White at APHIS has given us great continuity and a lot of guidance and support. Jim is also an expert in TMV, so that makes it a two-edged sword. You have a good audience, but you really have a good audience.

The good news is that in 1999 we produced several hundred tons of tobacco for processing on 32 acres, and we've received a policy statement from the USDA that we are good to go on a thousand acres of production material. This is no longer experimental material, and I think that's a very key word.

But during that time we have shown through rigorous field studies and rigorous analysis and very complex series of analyses and recordings that we have got good crop management practices. The virus, of course, does not go to winter. It does not persist in the field environment. We do a three-crop rotation, which is typical of tobacco growing and corn and beans. And these releases have been completely contained.

These are some of the things on our annual schedule. We have to notify the USDA every year and apply for not only movement permits but permits to release. We are inspected. We can be inspected at several times during the process, inoculation, structure of the field, and virtually anytime along the way to inspect our agronomic practices.

We also have to do a lot of post-termination monitoring of the crops in the fields. And we provide USDA-APHIS with an annual report.

Again, these are some of the issues that may or may not have to be validated in the field. And again, I'm not going to read the slide, but you can see that if any of these particular things -- let's take fertilizer, for example. If you show that you can affect the quality of your product through agronomic forces like MANO fertilizer or whatever, that's a validatable issue.

In the case of tobacco, we had not seen that as a determinant of product quality, but it could be. So each target and each protein that you're going to make has to have its own series of validation criteria run by it, and one size does not fit all.

Again, we are worried about vector development the same way a person would worry about maintaining a transgenic seed bank.

Same sorts of care and attention to detail for producing our virus for inoculant. Those protocols are well established and well written as far as our validation efforts.

Let's quickly go through some product examples. I showed you green fluorescent protein earlier. It's always a very spectacular one. It's also interesting to see what the

commercial version of it looks like, the context version of GFP on the right side and the tobacco-derived construct in the second line from the right. And this is done through relatively few chromatography steps.

I like this one. This is a viral conjugate showing three fusions to the coat protein of the virus. The reason that I like this one so much is that each one of those lanes represents 24 tons of tobacco. This is not a laboratory oddity, and not many people in business get to say that.

The line on the right is the wild-type virus, so you can see that there has been a conjugate made to the virus, but these represent enormous batches of material.

We do the same sorts of things that everybody else does. We look at endotoxin elemental composition, amino acid composition. We also look at small molecules because everybody is concerned about nicotine in tobacco, and we certainly will monitor that on a case-by-case issue.

We use the MALDI as an identity system, all of our product. Tryptic digest and Tryptic MALDI is another system that we use for identity and purity.

One of the products I did want to talk about a little bit was Alpha Gal. This is made by NEW PRO SYSTEM. And here it shows as one of the steps after several steps of chromatography, you can see that we are producing very clean product. To the right is the placenta-derived Alpha Gal that is currently being tested.

Specific activity, this number might not mean much to anybody, but let me just say that the specific activity of this particular enzyme, this Alpha Gal A which is derived from the human sequence, it certainly is not a humanized enzyme, and that's an area we can discuss in great detail at another time, but this specific activity is twice that of the normal human prep.

Probability is that the glycosylation of this particular enzyme is a very narrow range. If you look at human zyomoforms of enzymes, they have pretty broad gouging curve.

The case of plants, it's much narrower. In this case it might be better to be lucky than smart, and we are certainly going to take serendipity anytime it comes our way, and this particular construct is at about twice the specific activity of the normal human recovered enzyme.

I just put this up to remind ourselves that glycoform characterizations in plant-derived glycoproteins, therapeutic glycoproteins, is a mandatory part of the

identity and the purity of these molecules, and we will have to describe these in great detail. And I don't have time here to go into glycosylation issues, but let me say to you that you can become an expert on the total literature on the immunology or immunogenic properties of plant-based derived GLYC forms in about a weekend because there's very little literature on it. So again, the work needs to be done, and that's one thing that we are spending a lot of time and money on today.

Our release criteria, quality assurance: identity, purity, and safety. These are the three themes that you will encounter when you make that drug. And again, these are some of the tests and some of the quality assurance procedures that we use as a matter of course.

Another thing that we've done that is an aside, really, to this, but one of the things that we have done in Owensboro, Kentucky, and I think it's part of our new industry and is important to note, we've become very proactive as a company. There's two ways -- you know, you can go about this a number of ways to start introducing the idea of using recombinant materials to make therapeutics to the public, and we have taken it public.

We work very hard in our community. We sit on all three of the major universities' curriculum development boards and biology. We have an annual seminar series in Owensboro that we sponsor to bring in people from all over the ag biotech and biotech sector to talk about the uses and issues of genetic-modified materials.

We train our farmers. Our farmers are our real partners. The farmers are brilliant. There are no non-brilliant farmers left. You have to be very bright to be a farmer and tough as nails in this society to do this. We have great respect and we have great respect for the knowledge base of our farmer partners. So we spend an enormous amount of time with the farmers.

We're also very fair to the farmers. We tell them what the real time lines are, what the real issues are. For them to expect that they're going to have thousands and thousands of acres of tobacco being sucked up by multiple drugs within the next few years is ludicrous. We can't do it in the regulatory format fast enough to do it. The products are not that advanced.

But they're very patient. They're very futuristic. These people have the patience of Job and are sticking with us for the long haul, and we think that's a great union. We certainly do not want to become tobacco farmers.

I even sit on the Owensboro board of directors of the Owensboro Chamber of Commerce, so you can't get much more proactive than that.

So anyway, in conclusion, this is sort of how the thing is going on large scale. And we'll be happy to answer any of your questions after this is over, and I thank you for your attention.

KEITH WEBBER: I guess you can stay in the vicinity, and maybe we can get Carole Cramer up as well to answer any questions that the audience has.

If you can address your questions to one or the other or both. And if you have a question, as I said before, please go up to the microphone, and I'll call on questioners.

YASMIN THANAVALA: Question for Dr. Holtz. So I was mesmerized by the scale of your large scale biology, but you didn't address right until the end what one of the first speakers brought out, which is for vaccines, it's cost, cost, and cost.

So tell us what a product made by your method, say, the Fv protein single chain antibody, you anticipate would cost for clinical trial of a cancer patient versus a traditionally made antibody.

BARRY HOLTZ: The single chain is a very unique product. It's made patient specific, so obviously, large scale does not apply. However, our target is for less than $10,000 a year per patient. That's our target.

Now, that's going to require an enormous amount of automation and an enormous amount of process development, but that's a target.

YASMIN THANAVALA: Okay.

BARRY HOLTZ: The major lymphoma patient for years is probably hundreds of thousands.

YASMIN THANAVALA: Okay. Maybe I used a difficult for example but something like your Fabry Disease product. How much would that cost compared to the traditionally extracted --

BARRY HOLTZ: Well, the traditional one, if there is one available, if it follows the paradigm of GLUTENREANSITE, which is, what, between 3 and $400,000 a year per patient, we're targeting less than $40,000 a year per patient for that enzyme.

Was that clear enough? I won't share with you my operating cost, but I won't get that specific.

KEITH WEBBER: If I could ask a quick question. I had a couple, one for Carole, and let me -- You showed a graph with an increase in expression by your wound-induced promotor of the GUS protein after multiple mowings, and I was wondering if you could tell us, is that increased, say, at the fourth mowing, is that due to accumulative wounding effect, or is that just plant maturity?

CAROLE CRAMER: That actually wasn't an increase in GUS activity but actually a decrease in total soluble protein that is extracted. And so if you express those data as amount of GUS per fresh weight, it doesn't change. So every nucleus that's in the leaf is giving you the same amount.

What we found is in the last generation or the last mowing, the total protein that we extracted effectively out of the plant went down.

KEITH WEBBER: Which is the way to get the increase in specific expression.

CAROLE CRAMER: Yeah. We're seeing that was a specific increase activity demonstration.

KEITH WEBBER: And I had one question for Barry, and that is, with regard to some of the processes of transformation of this tobacco plants, what sort of parameters have you found that do affect protein expression or transformation that need to be validated?

BARRY HOLTZ: For the viral vector it's fairly straightforward. Our validation has to be in terms of our development and vector and make sure that the vector is what it's supposed to be, is delivered as is, as is advertised. We have very good fidelity in transport of the vector and use of the vector.

As far as the agronomic issues, fortunately at this point, we have not seen crop techniques affect the quality of the material. Now, I'll qualify that. It certainly affects the quantity of the material.

And I think to be fair, one should say that in processing, the quantity versus quality may be an issue in some processes. If you have very little quantity and you have to fish it out of a milieu of a plant that's very stressed or there is a problem, potentially that can be a problem. We haven't seen that yet, but we're certainly going to be aware of it.

GORDON MOORE: I was also interested in the issue of --

MADAM REPORTER: Could I have your name, please?

GORDON MOORE: Gordon Moore from Centocor. The situation is a little unusual because that's such an expensive protein. But maybe you could address the situation in the case of antibodies where there's a lot of them and there's a great deal of experience with their cost using conventional cell cultures so a comparison of your plant system.

And I guess I would address this to both speakers in terms of cost, the estimated cost to make an antibody which allows you to make a comparison between the plant system and the more conventional.

BARRY HOLTZ: Well, I'll comment on this in general terms. We produce large quantities of four different proteins in the Owensboro facility, so I'm quite sure of the numbers there, but the numbers that some people have glibly thrown around of, you know, $5 a gram proteins and things like that are not feasible, in my opinion.

If you're going to make a GMP-validated protein, your costs are going to be a lot higher than that. No matter if somebody hands you the protein for free, it's a raw material, it's going to cost more than that.

But to be in the hundreds to thousands of dollars a gram on some large-scale proteins is certainly within our range of capabilities, especially when we get into economies of large scale.

GORDON MOORE: So the most precise estimate you can give is hundreds to thousands of dollars per gram.

BARRY HOLTZ: Yeah, of purified protein.

JULIAN MA: Julian Ma. How do you protect your neighbors' farms from your tobacco mosaic virus?

BARRY HOLTZ: I'm sorry. Again.

JULIAN MA: How do you protect your neighbors' farms from your TMV?

BARRY HOLTZ: Well, it's very simple. TMV has got to be mechanically transmitted, so we segregate our fields. We clearly know where those fields are. We plant border rows of crops around them. We limit the access of machinery and people to the fields. And that's pretty much what you need to do.

It's not aerosol borne. It's not insect vectored. You really have to be fairly aggressive to infect a field.

Now, one particular advantage of being in burley country is that we don't grow burley. We grow FLEULAR varieties. But we grow in burley country, and most burley is N-gene resistant to TMV.

But we've never seen a case of transmission of the virus. Even in the case of the deer running through our field once didn't contaminate any other tobacco.

And I say we grow on a three-year rotation of corn and beans, so it's just good ag management. But that's a big problem. I mean when you look at a product that's made to require a thousand acres of tobacco to produce market supply, you really have 3,000 acres of tobacco in management at any one time, so the agriculture side of this is a formidable effort.

JIM FLINN: Jim Flinn with Bio-Endeavors International. We're listening here, seems to, about two different but same end purpose processes for making biopharmaceuticals from tobacco.

Would you like to comment -- I mean more than one case you inoculate in the field; in the other case you inoculate in a facility. Would you like to comment about -- Barry maybe first, since you're farther along -- which system or whether either system has -- what the advantages might be from a cost point of view from one versus the other?

In other words, do you see advantages to the field?

BARRY HOLTZ: I'm not selling stock right now, so that's not an issue, but there are advantages in all of these systems. And when we talked to our friends in the transgenics business, there are certainly advantages there.

The data and the products will out, I think, and when I talk to my friends from Monsanto in the corn business, there are obviously areas that they have good expression and especially monoclonal antibodies. Corn represents a big advantage. So it's on a product-by-product basis.

The big thing for us is that we can rapidly get constructs into production. It takes us about 10 days to produce a viral cassette with a new gene and get it on the plant. So we don't have to do traditional breeding.

We also have shown and demonstrated that in a very large scale, and I think with a lot of the other systems

right now, people just are not at large scale, so we don't know how robust they're going to be.

But I think if people do diligent efforts, there's going to be strengths and weaknesses in all these systems, and the market will allow it in the end.

Carole, you want to comment on it?

CAROLE CRAMER: I agree.

BARRY HOLTZ: Help me out here, Carole.

TOD STOLTZ: My name is Tod Stoltz. I'm from have a follow-up question to your virus escape question.

It seems that you've indicated that you've been monitoring your neighbors' fields or that you're monitoring the escape of the virus in some fashion. I am wondering if you can elaborate on how you've done that and if you can explain how you come up with the result that you were not seeing any escape of the virus.

BARRY HOLTZ: Well, there are several methods, and probably the most robust is PCR. When you can't PCR any information from soil samples or tissue samples that give you any forms of your recombinant material, it's either nonexistent, or you can't find it by PCR.

We also have very sensitive hosts that we can use to see if there are any viable materials left. We have antibody responses. We have a whole plethora of activities, and a lot of this, I think, is probably even published or is part of our reports at the end of the year. Those issues are not confidential business information, I don't think. Jim might correct me if I'm wrong. They're available.

And if you want to see the world's expert, talk to Steve Gardner afterwards because he's the one that writes all the reports, so he can tell you in great detail. I try to avoid the regulatory side of it as much as possible, but Steve is the expert.

JIM WHITE: I'm Jim White from USDA. And since 1991, when it was first field-tested, monitoring requirements, and tomorrow in my talk I will talk about biology of TMV and the data requirements that must be done.

Last year I went to the field sites when the plants were inoculated, and although the public perception may be the virus has a wide host range, that's true. Experimentally we can mechanically inoculate that in the lab, you can even infect, you know, 3 or 400 different plants.

But in the field it's biology as study, and it's only host where it persists in tobacco, and in tobacco production fields it's dead plants surviving in the field and going back to tobacco then next year and break that chain of command.

The other issue is tomato mosaic virus. Tomato mosaic, again, data will support that when you have tobacco mosaic viruses, tomato mosaic viruses, the same tomato plant, tomato mosaic virus, will all compete it routinely.

So there's data that's available. Some of that information, I think, is confidential. I shared that information before this meeting with John Hammond and Al Miller and, obviously, with BioSource's data reports.

And most of my recollection is the data on safety movement of the genes, the movement of the virus to weedy hosts that might be present in the field is publicly available.

One reason I went to the field was I wanted to see what weeds were in the field. These fields are remarkably clean of weedy hosts. Most of the weed hosts are not potential hosts for TMV. Most of them are grasses, which TMV doesn't infect. So I think there's a significant amount of data to show that TMV does not move outside of a field. Thanks.

KENT CROON: Kent Croon from Monsanto. A quick question for Carole. I noticed in the presentation you noted protein production is not largely affected by copy number, and you showed the range and one to three in terms of copy number.

The question I have is, did you see an effect in terms of insertion number? Did you look at all the number of inserts into the genome, or this is only copy number per insert?

CAROLE CRAMER: What we tend to do for every construct, because we use tobacco, it's so easy to generate lot of transgenics, so we routinely do 200 plants.

When they get to be 10 inches tall, we do a quick screen, wound induce, and look for a high expresser. So either we'll use an ELISA, a western, and identify those top plants and essentially throw the rest away because it's a hassle to carry these things on.

At that point we will look at copy number, and we routinely have among those usually at least half a single copy. So we primarily just take those single copy ones and move forward, and we demonstrate, so we do both segregation

but just some and demonstrate that it's a single copy to single site, and that's what we tend to go forward with and not even deal with any issues of what happens with multiple copies.

We routinely find that among our top three expressors, there's always single copy, and so we haven't sort of dealt with what are the issues of multiple copies. We've avoided the issue of multiple copies.

KENT CROON: I was curious this correlation with protein and number of inserts as well as you mentioned copy. Thank you.

KEITH WEBBER: I'd like to thank Dr. Cramer and Dr. Holtz for very educational presentations today.

And if everyone wants to go upstairs, there will be lunch served on the second floor, and at 1 o'clock we'll reconvene here for the next session.

(Short recess.)

CAROLYN DEAL: If everyone would move into their seats, we'd like to get started with the afternoon, please.

Well, I think appropriately for after lunch, we're switching topics from some of the purified biological products to what we hope is a new area of products that will have future developmental uses, and this is the area of whole vegetable or whole plant vaccines.

My name is Carolyn Deal. I'm from the Office of Vaccine Research and Review at the Center for Biologics Evaluation and Research of the FDA, and it's my pleasure this afternoon to introduce this section.

This is an exciting new area, I think, that's also a challenge for the agency because it combines a lot of technologies that we have not traditionally looked at in the evaluation of vaccines.

So it brings into areas of consideration all of the area of plants that we've heard about in the morning session as well as the things we traditionally look for in the evaluation of vaccines because when we license vaccines, we're obviously looking for issues of efficacy, purity, and potency.

And one of the challenges we talked about this morning is isolated biological products, but as we back up this afternoon and think about some of the whole vegetable-type things, how these could be of use as Dr. DiFabio said throughout the developing world but also some of the

challenging technical issues for how we would evaluate these products when we're looking at how we release these through the Center of Biologics.

So one of the things that we're quite interested in when we saw the publication of one of the first ones of looking at potato vaccines and delivering antigens for E. coli heat label enterotoxin in potatoes, and we of course immediately thought of the worldwide distribution system of McDonald's that could be coming into this market and what this would possibly bring.

But anyhow, that's kind of a lighthearted note of it, but it's my pleasure this afternoon. We have two speakers in the afternoon session that will address this issue. And what we'd like to do is take the questions at the end of the two presentations.

Dr. Koprowski is going to set the stage for some of the use of the vaccines, and then Dr. Richter will talk about some of the issues that I've just alluded to.

So our first speaker this afternoon is Dr. Hilary Koprowski. Dr. Koprowski is known for his long career in the development of vaccines against polio and rabies virus.

He was born in Poland and graduated from Warsaw University. He has served as the distinguished director of the WISTAR University for 35 years. And he is also a member of the U.S. National Academy of Sciences. And now he has the Center for Neurovirology at Thomas Jefferson University in Philadelphia.

So it's my pleasure to introduce this afternoon Dr. Koprowski who's going to talk about the green revolution in vaccines.

HILARY KOPROWSKI: Thank you very much. Mrs. Chairman, ladies and gentlemen, it's the worst time to give a talk right after lunch and particularly after lunch where you are served huge sums because I expect that half of you are already asleep, and the other will enjoy in a few moments a post-grand meal snooze.

So I do not know what to do wake you up. I've discussed with chairman should I shout, faint at the podium, do anything to call your attention. But I hope that since discussion will be held after two papers, you'll be sufficient awakened to ask question not in your dreams.

In the first slide -- first slide, please. I like to list my collaborators on the first slide rather than the last so you know they collaborated with me during the work which we'll present to you.

Dr. Dietzschold and Dr. Hooper are people who actually prepared some of the material for rabies vaccine. I should add that Dr. Rupprecht who sits in the audience has tested efficacy of the plant-derived vaccine in humans and mice, and Dr. Yusibov, another member of the Biotechnology Foundation, are instrumental in developing techniques and play a major role what I will present to you.

What you see in these slides is a little bit of repetition what Dr. DiFabio said, but I would like to call your attention to a few important items here. One is a question of one or two rather than multiple doses a vaccine should have. And I will tell you my personal experience once in Cairo, Egypt, when I visited the PASTEUR Center where they administer anti-rabies vaccine. At that time we had to receive 13 to 14 to 21 injections.

And the people who were bitten by rabid animals who wakens up usually disappeared after two or three injection and never returned. So even an injectable vaccine if given in multiple doses -- and here regretful I say that our efforts to combat AIDS by vaccination always directed in two or three or four vaccines in such content Africa will fail.

Now, the problem is, of course, the problem of cost. Now very important is that the cost of the vaccines today are somewhat astronomical, and nobody is to blame. It costs a lot to produce the vaccine. Now, that means that they are very limited in their possibility to be distributed worldwide.

Effective rabies is a wide variety of diseases. Of course, here we aim at the science fiction today that the single plant may be actually producing more than one antigen. I will show you it's feasible, but when it will be feasible to use single plant for producing multiple vaccine is still in the future.

Now, here I give you in examples of cost of the vaccines and maybe not very accurate, but essentially, you will see that in India – I give you India example. In India there are 50,000 cases of human rabies per year, and the vaccine which is available in modern new vaccine tissue culture vaccine will cost a treatment -- reduced rate would be $121, and the income of an average Indian per capita is $1,360.

Now, if he would have to have several treatments against rabies, obviously, he cannot afford such treatment, and we need to provide something much less expensive than the vaccine that's produced now.

Now, the remedy in India is that they have several small factories producing all time a vaccine which is 14 injection of a brain tissue. There's all these complications, and that cost about a dollar a dose, though I give you an example where we need a vaccine because of really too high cost for a given population. And you can read for yourself the same in Ethiopia, the really problems and the vaccine which needs only three or four injections is the cost in Uganda. I could probably multiply these countries by ten and still show you that we have no way.

Now, this is what we are trying to avoid, is harpoon by French cartoonist called Terra (phonetical), and it's called the black humor, and it's dedicated to physicians. If you have opportunity to look at this book, I assure you laugh from beginning to the end.

I picked up this because it really illustrates what I'm saying, this fear of numerous injection and, in addition, inability to have a personnel to administer injection and enough needles and syringes to be distributed.

So it comes to the point that they boil the syringe or boil the needle between one injection or another, and this is a terrible situation as far as administration. So I'm reading for Dr. DiFabio, the question that we need to consider administration of vaccine by different routes.

Now, this would be, of course, oral vaccine. They're easy to produce at low cost of delivery, best way to immunize, and there is a great safety for people who receive such vaccine instead of animal-derived products and also of those who prepare because you don't have to transmit anything from a plant to man.

This is a historical picture, and I decided I will show you our justification for the oral vaccine. This picture was taken 1958 in the then Belgium conga when live polio vaccine was administered to 250,000 children in 6 weeks and was administered orally and would be only possible to administer orally and prevented then an epidemic of true infantile paralysis polio affecting infants between 1 and 5 years of age.

Since then, as you know, the same oral vaccine made it instrumental to eradicate paralytic polio from this hemisphere, and this year 2000 will be probably the last stages of paralytic polio seen around the world.

So leading to eradication of polio it was only possible that we didn't use injectable vaccine but using oral vaccine which could be mass administered wherever your home.

Now, I use the term green revolution because in a meeting which took place in Brazil -- and many of you may remember when it happened, but I know that one of our presidents -- I don't remember who -- went to the meeting, was meeting about earth, and one subject, that was green revolution, and they listed the five acts of green revolution: plant breeding, soil and water, livestock, insect control, and environmental protection. And I have added plants as vehicles for biomedical products. So will be the six acts of green revolution or six projects of green revolution, and this is what my practice is.

Now, here is a thing which we talk all the time. I don't need to repeat again, the advantages of plants as production of delivery systems. And the facilities are easy in spite of I heard the last speaker that they are quite elaborate, but comparing what you have to go through to have vaccine produced in animal tissues, it's still much easier than in case of plant tissue.

Climatic conditions, the safety can be used as vehicle for oral delivery. I would also say that we speak about oral delivery, but one has to take consideration that we also consider at least from cost point of view would be intranasal delivery if we decided to give form of drops rather than food. Well, an important is inexpensive.

I do not know if this is exact calculation, but it is such enormous amount produced that it must be an estimated production one to ten of a dose, and even the adding manufacture, adding safety, adding everything, it will never match the cost of the present animal-origin vaccines.

In the Biotechnology Foundation where we are working, there are two systems considering how to produce vaccines. We essentially finally chosen the plant viruses, and I give you the very gross distinction of the difference in case of transgenic plants to integrate into plant genome to express the nucleus characters and is inherited.

In foreign gene -- and we're using plant viruses -- in foreign gene plant viruses is not integrated into plant genome, is located and expressed in cytoplasm, and is not inherited. And we have finally decided that to use mostly this approach because we thought that would be from point of view of purification easier and perhaps even a quicker method to produce vaccines.

Now, we use in our lab the alfalfa mosaic virions almost exclusively because they are very easy perhaps to manipulate, and they have some advantages which I will read to you. They are Plas RNA viruses. The particles are encapsulated and single-type protein. The size of particles

are dictated or determined by the size of virions RNA combined with them.

Independent of the size encapsulated RNA, alfalfa mosaic virus was formed lip or back-form particles. I will come to that to show you the difference when you use live virus and when you use modified virus.

Now, there are strategies to use to express foreign gene, and we essentially accept or help to present the advantage is gene replacement and gene insertion and implementation now more recently and more effectively than before.

Now, I would like perhaps -- is this focus? He was promising that you would focus it, and it would be no fault. It's getting more out of focus. Maybe it will disappear. All right.

This is alfalfa mosaic virus genome. It consists of four RNAs, and in this case they are called the four different properties. These are the replication, Replications 1 and 2. Here you have the movement brought in, and here you have a code property.

And what is done here is that we have obtained a defective replicate, and they were made by Dr. Ball in Denmark and even sent a P12 tobacco virus which has replicate, defective replicate, at five prime and remove the nucleotides so that it cannot participate in production of complete infectious virus.

And then into that transgenic replicate transgenic plant, we are adding a foreign gene, either linked with coat property or with the movement protein, and after it enters and using the subgenomic promotor, it's possible to obtain a virus which is not infectious. There are particles unseen, but it cannot transmit it, so it's a bioquantitation, is bioprotective system. It cannot spread from one plant to another, and that was the advantage of this system.

Here you have again the same explanation. Maybe we can focus it. All right. But again you have the same plant which I mentioned to you which is transgenic for Replicate 1 and 2, and we come with our constructs here of foreign gene or whatever you want to do, link this movement, in this case could be linked to the effective events occurring inside the glass. Therefore, there is no inherit and there is no way to transmit the virus from one to another, one plant to another.

Now, here is a electromicroscopy of live alfalfa mosaic virus. If it would be in focus, you would see there

are cylindrical particles, and there are also spherical particles in the live virus.

When you have a five-prime isolate preparation, isolated already together with the foreign antigen and in the plant that's transgenic for replication, you'll notice extremely few elevated particles and a lot of spherical particles which represent a noninfectious material.

And this shows and this is another virus agent. And knowledge of recovery of infectious virus recall the system implementation, and this is P12 transgenic tobacco for replicate. And attention made to recovery of infectious virus making a second passage of this material into two of tobacco and spinach.

And you can see it is transitional virus expressed in the plant, and here is some stop, a hormone expressed in the plant. It is after five passages, stability of expression of no recovery of infectious virus from the material in the course of the five passages. So material is stable after passage -- can be passage of plant to plant, or you can always a new seed lot to infect plants, but you can be assured that it doesn't spread any further.

Now, here is a beautiful spinach leaves which carry, in this particular case, rabies antigen.

Now, there is immediately when we start talking about plant viruses, then I don't know how to classify this people about this lunatic fringe which always finds some fault with anything you do. They are objecting transgenic plants, and now possibly they will object to the virus for the viruses.

So I had to find some argument because disputed scientific arguments won't help, so we found a paper here where curious people surveyed peppers from stores in this location in California, and I just see you the number of viruses which you have recovered from these peppers, and I assure you if you had salad today, you had your portion of plant viruses, and looking at you, you seem to remain very healthy, no side effect after this meal.

So this will, I'm sure, convince somebody that since you eat plant viruses all the time in your life that if you'll be eating such virus together with some other antigen, you will be safe, and you won't die terrible death after your first feeding.

Now, here are results of some of the expressed biomedicals in plants. Now, in lettuce Hepatitis B virus, I will just turn to immediately, and it was not purified. It was given in form of a lettuce expressing Hepatitis B, and this is the quantity which a fresh leaf would show.

Rabies virus was spinach and tobacco. It can be purified and was again quantity of ten. Respiratory syncytia virus, I don't expect to give you all lecture on virology, so I'm sure you know what Hepatitis B virus is, and probably you know what rabies. You may be more difficult respiratory syncytia.

It is a disease of newborn, rather a dangerous disease which causes death among many newborn and for which there doesn't exist an effective vaccine today. Therefore, we thought that here would be ideal material, a plant material which would be incorporated in baby food. Now, that will remain to be seen, but we still have to do some work.

GA-733 is colorectal cancer antigen which I have myself discovered was many, many years ago, and this has been purified with tobacco. And next we have Ure A, and Ure A is attempt Ure A to put in plants in this case in carrots because it will immunize against Lyme disease, and it's a pretty good antigen.

And this is an antibody, 17-1N, which my colleagues and I have first described years ago, and it is an antigen antibody recognizing an antigen on colointestinal tumor cancer, and it has been used extensively in a clinical trial in Germany by 30 percent of people who receive this antibody for injection that 30 percent less metastasis and 30 percent survival rate in comparison with the others. So we decided to use this material.

HIV was more used for play because we don't have the money nor experience nor patience to go into this big milieu with HIV and try to show that we can grow in plants. So I will show you as a curiosity.

Now, here we are discussing efficacy. Now, Hepatitis B virus was expressed lettuce. It was used parenteral in mice and oral in mice and in human. Rabies virus in spinach, immunized mice parenterally, orally, and there is a trial now discussed with human.

Respiratory syncytia virus, we have a good immunization in mice, and AIDS, HIV, one of the things we looked at it has been expressed in tobacco and has immunized mice but didn't really go any further and will not go unless our personnel increase.

Now, these results we obtained in a bioorganic institute in city of COZE in Poland who collaborates with us, and these are results of theory of lettuce expressing Hepatitis B virus in this stage of the three volunteers.

And you will notice in two out of three, you had very strong, 150 grams of lettuce twice or three times, very strong antibody response. The antibody level corresponded to those levels which protected against disease.

Now, the question -- this is a very important trial, and it is now repeated in 20 volume here non-Hepatitis, was the same results because it shows one thing which bothered all of us all the time until this was done. If we eat lettuce every day, then theoretically may not be able to develop antibodies and may be what immunologists would call tolerant administration of lettuce.

Therefore, it was very important to find out that this is not the case, that if lettuce contains foreign antigen, it's still capable to immunize people, and this is probably the first time this was demonstrated and given great encouragement to people who work in this field that it doesn't matter, that they can use food as carriers in expression of vaccines and on any biological products because even though this is a stable diet for them, they still will not be tolerant and can develop antibodies, even high titer.

And this has been repeated by these people, and 20 volunteers felt again 150 grams of lettuce expressing Hepatitis B with the same results. They develop antibodies.

So the chart is small, but importance of the chart is in the fact that you may immunize people by oral feeding of a staple food expressing foreign antigen.

Now, this is immunization with rabies of mice. It's parental immunization and then the results of challenge. Now, results of challenge indicate that -- and these are for nonimmunized. They all died. It's lethal challenge rabies virus, and out of these four mice died, and six survived, late death, much later intubation period shown here in days than of controls.

We will now forewarning to Dr. Rupprecht next week and more spinach-expressing rabies virus antigen, and we hope that he will feed them to dogs and find out in the case of dogs all the feeding will produce antibodies. I will tell you later about human trial.

Now, here is oral immune response of mucosal IgA in mice after immunization with recombinant of rabies LYPOPROTEIN with the plant.

Here is a very interesting trial, and I would like to say a few words because it's complicated. When after negotiations of the year, I would say a year negotiation probably, of being able to feed spinach with rabies virus to

humans, we have agreed, and perhaps justified, that the first feeding would be done to people only the immunized these. So you will evaluate the safety of the procedure minus rabies safety. And this was done.

Well, of course we paid the price of doing this experiment on individuals because following first three feeding of spinach-expressing rabies virus, we didn't find any difference in level of antibodies.

But then we came, and we subjected these people to commercial vaccine, and here came a very interesting results. This here are again sent to Dr. Ruprecht, and Dr. Rupprecht gave us the results. These are the experimental means dose were fed rabies, spinach with rabies, and those were spinach alone.

In this case only one showed a remarkable boost in his antibodies as compared to controls, but in case of people who before were fed rabies spinach, the booster was remarkable and significant.

So now we are feeding volunteers who are not immunized previously to rabies, and we hope that this will really indicate that the feeding of such type of spinach in two or three feedings will give us results.

We have not committed ourselves yet to the dosage. The dosage will depend whether should there be one feeding, there should be constant feeding, or three or five feedings. We have chosen the dosage of some positive and will have to be determined what happens with one feeding with small amount because there is a breaking of tolerance more superior to five feeding, or should we three feeding, you need then to conduct a thorough clinical trial, and probably we will not conduct it, but some will be interesting.

Now, these are respiratory syncytia virus. Respiratory syncytia virus may constructs in tobacco and by the implementation method, and this is the dosage antigen used to immunize mice.

Serum titers are very high, and mice can be infected with respiratory syncytia virus. And determination where they were protected is made on potency and on examinations, the logical examination of lungs.

In the case of controls, most of the lung tissue contained virus which can be visible even of certain ANTOLOGY, and in the case of immunization, you can see that three out of the -- and four of the four mice showed that they were immunized with respiratory syncytia virus.

This work will progress probably by putting another antigen -- this was of a respiratory. We put G protein, and then we will see by that time the fear of feeding plant material to infants with a biologic will still be problems. This is actually curiosity. I told you that we are preparing HIV in plants and tobacco and here for immunized mice and show you that the mice develop antibodies against HIV.

Now, speaking of HIV, the only advantage of plant, if you'd be able to use plant to produce what is so-called different class of the HIV so that you will be covering a large immunogenic component of the virus by immunization. But again, as I said, it's a probably very tempting proposal to do but will have to be done by much bigger sources than we have.

Now we are coming to this antibody history which I showed you which is now licensed in Germany for the use with great success. We decided we will use it because possibly by producing it in plants, we will decrease the cost of the antibody which can be used in quite large quantities by being used with about four injections after operation.

Now, in this case the system is still very similar aimed at the light chain is still virus, and the head chain was another protein and both of them used to infect the same plant which produced in the case a complete antibody.

And again, it is the same transgenic replicate transgenic plant in order to prevent formation of complete homozygous.

And here are the results of this test. We can see that we have detected full-length antibody after infecting the plants. This was done in tobacco, and here are the simple chains, that light inhibit chain, and this is a complete antibody.

So this has been shown by putting from two viral constants from code parting, there is movement parting. One was heavy chain; one was light chain. A complete antibody can be produced, and that antibody is a plant from which you can transmit the mosaic virus.

A very strange occurrence. This is the plant-derived 17-1A antibody, and this is the commercially produced 17-1A. This one is much greater affinity. Now, this needs to be confirmed, and of course, we will have to do the functional tests on the antibody. The functional test in case of colorectal antibodies, implantation of colorectal concept under the mice and then treat it with antibody.

In this case this antibody prevent growth of the tumor, and we expect that perhaps the same happen as the antibody is now occluding sufficient material in order to be used in functional antibody dose.

This is an analysis of a light chain of the same antibody plant-derived, and here is the standard antibody, and here is the light chain of the plant antibody. These are markers.

Well, finally, I have mentioned to you that in case of one plant, it is possible to probably produce antigenic expression of several antigens and not only several antigens but several combinations.

Probably one of the tempting combinations will be putting the same plant GNC of F, which is induced in antibody inducing numerous response which they've given vaccine or antigen.

In this case, however, what was done is a combination of the HIV GP-120 particles and rabies. They were put in the same plant, and here you have the V3 loop and the antigen show rabies virus, and you can see that both were expressed.

We have not pursued it anymore again. It is just to tempt your appetite that there is possibility to develop this, and this would be very valuable for immunization and for vaccination for even administration of hormones or drugs.

So now I just present you my last slide, the new logo for this period. All right? Before it was Pasteur. Then you're convinced as children to Popeye, convince you to eat spinach. Now you eat spinach and have results as Pasteur.

Thank you very much.

CAROLYN DEAL: Thank you very much, Dr. Koprowski. As he's illustrated, some of the tempting new approaches for this technology also brings up some very practical questions for all of those of us in the manufacturing industries and the evaluation industries.

Dr. Richter is going to talk to us next about potency, consistency, and stability of some of these products. Dr. Richter got her Ph.D. from Baylor College of Medicine, and she did a post-doctoral fellowship with Dr. Charles Arntzen, and she's currently a research associate at Boyce Thompson Institute.

Dr. Richter.

LIZ RICHTER: Good afternoon. Well, thank you, Carolyn, for inviting me to give this talk.

When I first joined the research group of Dr. Hugh Mason and Dr. Charles Arntzen five years ago, they were about to publish the first proof of concept studies on edible vaccines. That first paper described mouse feeding segments in which they simply fed mice tuber material, raw tuber material, that expressed the heat labile enterotoxin LT-B subunit.

Those studies were successful, and since that time, several other groups have done animal studies and even clinical trials. So there has been a lot of interest in this area of edible vaccines, and I think one of the true strengths of the plant as a production system for pharmaceuticals and proteins will be in the area where you can have oral application, where you don't need to have a purified product.

And that's what I want to talk to you about today, is some of the practical aspects of producing something in plants for oral application. Can I have the first slide?

There are quite a few new therapeutics under development, but not as many as we would like are being developed for oral delivery. There are some constraints against oral delivery. You have to be able -- or the pharmaceutical has to be able to survive the digestive environment of the stomach, and this may require more protein than if you were to deliver through injection.

We've heard discussion about safety considerations are important for oral delivery, the cost of equipment and personnel, compliance.

One thing that I haven't heard mentioned today is that if you deliver a vaccine orally, it may be possible to induce both mucosal antibodies and serum antibodies. That may be important for diseases that infect through the mucosa. You may induce the first line of defense against those diseases.

But delivering edible vaccines, these products will not be considered a dietary food. They are vaccines. They are medical products for which the dosage levels and timing of immunizations must be controlled.

Boosting strategies will have to be worked out for each individual vaccine antigen, and the use must be guided by public health professionals.

I'm going to talk about three areas of which potency is the first one. Several factors can influence the potency

of an oral vaccine: the amount of antigen that's delivered that may survive through the stomach to get into the intestinal tract where you have immune tissue that can uptake that antigen.

The antigen form may aid in that survival through the digestive tract. And also, the antigen form may be very important in whether or not the immune cells will recognize and take up that antigen.

Whether or not there is an adjuvant that is delivered with the vaccine can be very important. The pH sensitivity. Again, this addresses, will it survive the trip through the stomach to get into the intestinal tract? And resistance to stomach digestion. All of these things are considerations when you're trying to make an oral vaccine.

I'm going to give you examples of three ongoing projects from our research group. The first example is the vaccine against travelers' diarrhea composed of the B subunit of the heat labile enterotoxin

The B subunit forms a pendular as shown here, and that pendular combines to GM-1 gangliosides. These gangliosides are on the epithelial cells that line your intestinal tract.

So in this experiment the LT-B subunit was expressed in tuber material, and it was expressed at a level of three to four micrograms of B subunit with an extra signal at the carboxy terminus that would allow that protein to be retained within the endoplasmic reticulum.

So it was expressed at three to four micrograms per gram of raw tuber. The mice were fed 5 grams of tuber on Day 0, 4, 14, and 18 for a total of only 80 micrograms of antigenic material.

This graph shows in the black the IgA response. The white bars are the IgG response of mice that are fed different transgenic potatoes. In each case mice did respond with both serum and mucosal antibodies against the LT-B molecules, and that's compared to a bacterially produced recombinant LT-B shown here. So this was that first proof of concept experiment that was published, and here we had only 80 micrograms of the antigen.

Those experiments used potatoes shown in the center here that expressed .01 percent of their total protein as the LT-B subunit. Since that time, our group has made synthetic gene that allows a lot better translation within the plant cells, and so now we can make up to .5 percent of the total protein in the tubers being the LT-B molecule.

So those first mice were fed tubers that expressed 2 to 5 micrograms per gram tuber. Now we've increased the potency to 10 to 20 micrograms per gram tuber. So these type of experiments where we increase the antigen level presumably will increase the potency of the edible vaccine.

Another example I want to talk about is the Hepatitis B vaccine. Here we're using the Hepatitis B surface antigen protein. This is the same protein that is produced in a yeast system and sold commercially for an injectable vaccine.

If we want to compare plant production to yeast expression, plants will disulfide bond the Hepatitis B surface antigen whereas in yeast, the monomers must be purified and then processed to have disulfide bonding.

Plants will also glycosylate protein. With scale-up agricultural practices can be used. We are working on an oral vaccine instead of a yeast-injected vaccine. We're using partial food processing versus purification from the yeast.

Our initial mouse studies for oral delivery started with tubers that used 1.1 microgram surface antigen per gram of tuber. The mice were again fed 5 grams per dose, and they were given a total of three doses on Day 0, 7, and 14 for a total of only 16.5 micrograms.

This is the antibody response in MILLI-INTERNATIONAL units per ml serum, and this is the weeks after being fed. So they were fed at Week 0, Week 1, and Week 2. And then we see an antibody response in the serum where the antibodies go up to 75 to 80 MILL-INTERNATIONAL units.

And it's a short-lived response. It comes back down, as we saw with Dr. Koprowski's human data. The response did not last that long, but that may be because the antigen level is very low. If you consider that the injected vaccine against Hepatitis B uses 10 micrograms, and that's injected, if you're using the oral route, you may need 10 or 100 times as much material to get a comparable immune response.

At Week 10 we wanted to determine whether the immune memory had been primed, and so we gave a suboptimal boost in the intraperitoneal of .5 micrograms of the commercial vaccine, and here we see an immediate high titer response.

So this shows that the immune memory has been stimulated, and we can get a primary response, although it is short-lasting. So perhaps for the potency of this particular edible vaccine, it's very important to increase that amount of antigen for our initial dosage.

And working towards that end, we started with tubers that produced 1.1 microgram per gram fresh tuber. We've been able to increase that to about 10 micrograms per gram in fresh tubers.

When I moved to tomatoes, my initial levels were around 10 micrograms per gram, and this is using a normal-size tomato that might be used for food processing. It's a commercial variety that could be used to make tomato paste pictured here from our greenhouse.

When I moved to a micro-tomato variety, my levels increased to at least 40 micrograms per gram. And here's a picture of the micro-tomatoes. These are smaller than cherry tomatoes.

And there might be several reasons that the micro-tomatoes have a higher level of transgenic protein than the normal tomatoes. There certainly is less liquid in these tomatoes. There's a lot of meat compared to liquid in them.

We're currently crossing some of the high-expressing micro-tomatoes to normal tomato plants to see if we can get a very high-expressing normal tomato. But all of the further studies I'm going to describe in tomatoes have been done with the larger tomatoes. I've only just started working with the micro-tomatoes because from any individual plant, you don't get very much material to work with.

This is a picture of one of our tomato greenhouses, Boyce Thompson Institute. Some of the advantages of tomato versus potato, we started -- well, actually Dr. Arntzen and Dr. Mason before I joined the group started with tobacco and then moved to potato as a model system because the raw tuber could be fed to mice and humans for studies.

From there we've moved to tomatoes, and some of the advantages are that tomatoes are eaten raw by humans. They can be grown in containment, grown in greenhouses. We can use genetic crossing to combine the different vaccines. We can also do genetic crosses to make male sterile plants.

We can cross them into varieties that have identifying colors. I understand that there's a rather unappetizing white-colored tomato that might be good for a medicinal tomato. I don't want these edible vaccines to be taken for simply crops. We want people not to eat them except in the correct regimen. Tomatoes can also be easily processed, and I'll describe this in a later part of my talk.

Another aspect of potency for the vaccines might be their form, whether the antigen can assemble into multimeric forms. And here I'm showing a western blot of the Hepatitis

B surface antigen. On the left these have been boiled for 20 minutes in the process of 100 mill DTT.

On the right these were heated to only 60 degrees for five minutes. The center two lanes are 50 nanograms of the yeast-derived Hepatitis B surface antigen whereas the outer lanes are just a crude tomato extract from the micro-tomatoes that are expressing the surface antigen protein.

So we can see that if you boil the yeast material, you'd have mostly monomers, some dimers and a few trimers. The plant material looks very similar in that you have mostly monomers, some dimers, some trimers, and you can see a few larger multimers of the antigen.

If we heat it only at 60 degrees, we see this ladder of subunits going up even higher. So the plant material is ALIZAMERIZING these monomer surface antigen proteins, and that can be very important for the potency of the ADB vaccine, especially if it's going to have to survive through the digestive tract.

This panel shows results from SUCROSE gradient where a crude extract of the tomato, the extract was run on a SUCROSE gradient to try to determine how large were these multimers. Were they indeed forming virus-like particles that would be similar to the yeast-derived material that's injected for the vaccine?

And can't really read this, but at least one of these is the positive control of the yeast-derived particles, and the other samples include sample from fresh tomato or lyophilized tomato. However, leaf extracts, dehydrated tomato material.

In all of them we can see that virus-like particles are formed, and they're very similar to what is found in the yeast-produced vaccine.

So I want to move on to consistency of antigens produced in whole fruit or vegetables. And here you've heard a little bit of information on this from Dr. Russell and Dr. Cramer about production in corn, how consistent from generation to generation and crop to crop and within a crop the production of the recombinant protein is.

So what we found is that for consistency of antigen, the health of the plants can greatly affect, and this is more true for potato than tomato. With potato if the health of the plant has not been very good, then for the Hepatitis B vaccine, you do not get a very good level of antigen.

There are many factors that can affect the health of the plants, and as we've had more experience with growing

them, we are becoming more consistent with the health of the plants, and we've been able to produce more consistent batches of crops.

The pests that are on the plants can have an effect. Whether you're using sprayed pesticides or biological controls can be important. The growing seasons, growing conditions in the seasons, can make an effect on at least the potato that I'm using.

The tomatoes, what I've found recently is that trying to grow tomatoes, even with supplemental lighting, I get a decrease in yield over the winter. It has been very dark this winter, so we've not had as much light as we would like.

We still get tomatoes that produce a good level of vaccine, but we just don't get as much tomato mass as we could if they were grown during a different season. And that may not make any difference if you're growing them in a different part of the world where it's sunnier during the winter. We seem to have winter about nine months out of the year.

The genetic background can be very important when you're looking at consistency of production of a transgenic protein in a crop. We started with a potato variety that is not used for commercial purposes. And now we have moved into a different variety that is harder to transform. It takes longer to transform. But hopefully, it will have more consistent production.

This graph shows the amount of Hepatitis B surface antigen from tubers that were harvested from several different pots from one crop. So all of these bars represent an individual tuber.

These are all the same transgenic line. They were grown at the same time in the same greenhouse but different pots. And you see a variation, the average of 4.14 and a variation of.94. So 25 percent variation from tuber to tuber, pot to pot.

When I say that would be one crop, that would be potatoes that are all planted at the same time and harvested at the same time. So you can see here's some pots that have been planted more recently than these potatoes, so if these pots were growing the same line as these, this would be considered a different crop of that particular line.

And I want to show you some data from the same line but different crops now. And this is showing results from tubers that produce the LT-B antigen. Here we have different harvests but using the same transgenic line, so

each group of bars represents a different harvest, and there were several different tubers harvested. Actually, two tubers from this harvest was assayed. One tuber was assayed, two tubers.

And the third example of an antigen produced in transgenic potatoes that I want to show you uses Norwalk virus Capsid protein. The capsid protein has been expressed in the baculo virus system and can form a virus-like particle, and it's been shown to be stable at a pH of three.

In the plant-derived Norwalk virus capsid particles, the plant-derived recombinant Norwalk virus capsid protein mimic the baculo —virus-derived material, including the formation of virus-like particles.

So here I'm showing you a bar graph of different transgenic lines, and several of the tubers for each line are from different harvests. I'm sorry. This is tomatoes.

So this bar would represent one transgenic line and twenty different tomatoes. Sorry. You probably can't read these numbers here, but for this one there's a standard deviation bar, and twenty different tomatoes were assayed.

Those twenty tomatoes were picked from different plants from different harvests, so you can see the consistency of tomatoes through different transgenic lines. So this particular line, we've only tested one tomato. Also for this line we've only tested one tomato, but some of the others, here we've tested thirteen tomatoes, and the standard deviation is here. This was five tomatoes with a smaller standard deviation. Eight tomatoes. This is a larger standard deviation. Three tomatoes and six tomatoes were tested.

I'm moving into doing some ripening studies to test for consistency of production of antigens in the tomatoes. This particular line of Hepatitis B surface antigen tomato uses a 35S promotor. This promotor should express in most plant tissues, and it should be expressing even in the green tomato.

So when I assayed these tomatoes, I picked them all at the same time and did the assay at the same time. Unfortunately, the green tomato is very hard, and I just use a blender in the laboratory to grind up the material, and I didn't realize until I was already pouring the material out of the blender there were a lot of chunks left that were not homogenized.

Up until that time I had been using the ripe tomatoes, and if you blenderize a ripe tomato, it is made into juice and puree within, you know, five seconds whereas the green

tomato is very hard. And so unfortunately, I think this data point is not correct, and I'll have to repeat that.

But if you look at the breaker stage tomato and the orange tomatoes and ripe tomatoes, you see that the level of antigen expression is fairly consistent for this particular line of tomatoes.

I've gone on to do some further studies with very ripe tomatoes. These tomatoes can be picked ripe and kept on the lab bench at room temperature for at least two weeks, and I still see a good level of antigen production within the tomato.

Of course, you keep a ripe tomato at room temperature for too long, it's going to start turning soft and squishy, and at that point you do see a drastic reduction in the antigen level, so certainly want to use our tomatoes in the ripe stage but not overripe stage.

So that leads into a discussion of stability of the vaccines and what affects that stability. The type of fruit or vegetable that you're using in potatoes can be very stable, and I'll show you data from that in a minute versus tomatoes which you can't keep sitting around for very long.

The storage conditions, if you want to keep the potatoes at 4 degrees versus keeping them at room temperature or if you want to try to put the tomatoes under refrigeration, that might have an effect on the protein content. And then finally, whether you're trying to use a whole fruit or vegetable versus a processed fruit or vegetable.

This graph shows in the black bars the amount of antigen in Hepatitis B surface antigen tubers when tubers were first harvested from each pot. Then nine months later I assayed two more tubers, and those are shown in the white bars.

So with nine months of sitting in the cold room, these tubers actually had more antigen level after being stored, and there are several possibilities as to why they measured a higher level of antigen.

The tubers do dehydrate somewhat upon storage. It could be that the antigen monomers will assemble into dimers with storage. It may be that the antigen is more extractable upon storing the tuber.

The tuber is still a living entity. It is still producing proteins. And it could be that the 35S promotor is still cranking away, although very slowly, so that there is a higher accumulation of the surface antigen protein.

Tubers seem to be very good for storing the proteins, at least up to nine months. Now, after a couple of years, three years, when I've looked at some of our stored tubers, they're really shriveled little unappetizing things, so I don't think that you could keep tubers much longer than a year and have them look like you'd want to eat them.

So moving more into partial food processing, I think these are very applicable to the edible vaccines. We might be able to use things such as freeze drying, dehydrating, juicing as with the tomatoes, spray drying, or pulping.

These are some pictures of juicing the tomato NTB vaccine. Here I've just picked several tomatoes and tend to grind them up with a hand blender on ice. I strain the juice to remove the seeds and peel. I keep the seeds. And I weigh the juice.

Some of juice I will freeze, and then I'll lyophilize. This takes one to three days to lyophilize anywhere from 50 to 100 grams, and I'll end up with a freeze-dried powder.

Now, through this process when I measure the antigen levels on the powder, I end up with about a 50 percent loss of measurable antigen. But I still end up with levels up to 100 micrograms per gram of powder.

If I want to try dehydrating the juice, what we do is we use just a food dehydrator that we bought from a local store. We put it in the cold room because it doesn't have any temperature controls. When we had it at room temperature, it went to 70 degrees, and one or two days of heating the tomato juice to 70 degrees has a detrimental effect on the level of the antigens I'm working with, and that's going to vary for whichever protein you're trying to express. But for the Hepatitis B surface antigen, that much heat for that long does drastically decrease the antigen levels.

Whereas when we put it in the cold room, it heats to 50 degrees for 24 hours. And I see a little more loss with the flakes than I do in the powder. But I'm still getting very high levels of antigen, say, up to 60 micrograms per gram in these processed flakes.

So with partial processing techniques where I have not added anything to this tomato, I've simply juiced it and then processed it, I've been able to make a stable dry preparation which was allow batch production, consistency of dose, microbial testing, individual dose packaging, and long-term storage.

My first lyophilized powder was made several months ago last fall, and every time I've tested it, it has remained stable. It is not losing anything with storage at room temperature on the back of my bench.

Now, none of this has been optimized other than moving that food dehydrator into the cold room to reduce the temperature, so I think certainly with the Hepatitis B surface antigen, if we were to use a spray dryer where the juice is sprayed out and as it falls the little droplets dry into powder, there it's not exposed to heat for very long, and I think that would increase the level of antigen that survives through this process.

So for any individual protein that you want to do a simple processing step, you're going to have to use techniques that are appropriate for it to survive that processing. You know, whether it's heat stable or not will be important.

So I just want to come back to my original slide and say that these edible vaccines will not be considered dietary foods, that they are medical products, and our current strategy -- instead of giving fresh tomatoes or raw potatoes for the vaccine, our current strategy is to use dry food samples or powders or concentrated extracts for adults and children and then possibly a food puree for infants.

And our overall goal has been to develop new technology for vaccine manufacture via adaptation of agriculture and food industries, sort of a modern herbal medicine.

Thank you.

CAROLYN DEAL: Thank you, Dr. Richter.

We'd like to open this session up for questions. If you would come to the microphones, have questions for Dr. Koprowski or Dr. Richter. I think that we've seen an example today of some very early results in this area and some exciting new approaches.

WALTER GOLDSTEIN: Thank you. Walter Goldstein from Biolex, North Carolina. I have a question. It relates to another abstract of physiology in response of antigens or materials when they pass into the gastrointestinal tract beyond the stomach and they enter the mucosal layer, more response or passage in some tissue. There can be response that can be like a side effect besides the beneficial response. It also could be a beneficial response.

So I'm interested in the kind of studies that might be conducted to examine that to make sure that the gastrointestinal tract, you know, maintains because of the nerve endings and all sorts of the supplements that are involved in that part of the -- either speaker. Thank you.

CAROLYN DEAL: Dr. Koprowski, would you like to address that question?

HILARY KOPROWSKI: If I understand this long question, you're really asking whether there is an infection in the gastrointestinal mucosa?

WALTER GOLDSTEIN: I'm interested in a manifestation --

HILARY KOPROWSKI: When we do biointestinal tumor and find that there are any changes, it was not done.

WALTER GOLDSTEIN: Okay. It may be -- like, it could be an inflammatory response, but it also might be just a response that you can't figure out why no special beneficial as well.

HILARY KOPROWSKI: All I can tell you, that no whatsoever gastrointestinal symptoms following repeated heating, but I can't answer your question that we saw no mucosal.

Since I have the microphone, I might be able to tell you one more thing. That -- sorry. I'll be done in a second -- that there are two --

CAROLYN DEAL: Would you like to come to the podium?

HILARY KOPROWSKI: I'll tell you one thing. There are two immune systems essentially in the oral tract, and we should not overlook them. One is the saliva pockets, which of course is our target, but the other are tonsils.

And what you should not overlook possibility that if you find in saliva antibodies following feeding of this material that it is from the tonsils, the response of the tonsils.

So it's not only the problem that it goes through gastric tract, but also it may be rather upper intestinal tract. Upper tract, it may produce vaccine. We have seen it before in other antigens.

WALTER GOLDSTEIN: Thank you.

BRUCE CARTER: My turn?

CAROLYN DEAL: Yeah.

BRUCE CARTER: This is for Dr. Koprowski also. I was wondering in your spinach rabies -- I'm Bruce Carter. I'm with CVB, Center for Veterinary Biologics. And I was wondering for your spinach rabies Glyco Protein G vaccine if you intend that eventually to be a primary vaccination that would require a booster with, let's say with human diploid cell vaccine, or do you think that there would be a rapid enough anamnestic response that you would not necessarily have to booster with it, and if that is the case, do you think that you would ever conduct an animal vaccination challenge study to see if feeding spinach alone would protect people – be protected?

HILARY KOPROWSKI: The first question is question which I ask myself. I don't know whether it be primary -- primary and then booster vaccine was needed either vice versa or it will be sufficient. We don't know that.

As far as challenge, Dr. Rupprecht who sits here in the second row, the antibody challenge adults, so we will know about it. The first question is a very good question.

And my colleague, establishing memory, or is it actually protected? And this is very good problem which we will have to investigate.

BRUCE CARTER: I don't know how much good this is doing. Thank you.

CAROLYN DEAL: Actually, Dr. Richter, I had a question. Do you have to change the gene sequence any to get the plant code on usage more appropriate for any of the genes that you've expressed in the plants?

LIZ RICHTER: Yes. For the LT-B gene it made a big difference producing a synthetic gene. For each of our antigens, we now analyze the genetic code and look for cryptic plant signals as well as code on usage, and some of the antigens are somewhat plant friendly; others are not.

For the ones that are not that don't look like they would express well in plant cells, we make synthetic genes now.

GUY CARDINEAU: I have a question for both of you. I'm Guy Cardineau. I'm particularly concerned about dosage, and so I had a question for you, Dr. Koprowski, with regard to the rabies dosage.

You mentioned several different dosage regimens that you're considering, and I was just a little curious about that. I know maybe Hugh was going to talk about this

tomorrow. With the Norwalk virus trial, there were different dosage regimens, and there seem to be a different response whether you dose twice or three times. So that was Question No. 1.

And Question No. 2 for Dr. Richter was with regard to the primary response in your -- I think it was the Hep trial versus the secondary response boosting. It would seem to me that memory is perhaps more important than the initial response, and I'd like to get your feeling on that because that's what we're really looking for. You're going to vaccinate somebody, and sometime down the road they're going to get infected, and it's the memory that's the critical issue.

HILARY KOPROWSKI: I have mentioned that it is a task to establish proper dosage, and we will have not to base it on mice. You have to do it what happens in man because this is a different story.

So we will try -- we tried one type of dosage essentially by 150 grams of fresh leaves as one dose and repeat it in case of hepatitis, I think, twice or three times in case of rabies. We fed ourselves three times, bleeding after -- collecting blood after each feeding.

So you will get information from that trial whether one feeding is sufficient or whether three is sufficient or insufficient. That will give some information.

The thing which goes always in my mind, and I will frankly share it with you, is that because we are dealing with that semi-tolerance situation maybe we need to do one small dose in all these dose. This we will try in mice, and then we will have to come to man and try again.

So at present we use the same dosage, but I will be the first to admit this is just taken from indication how you immunize orally. Please remember there are very few oral vaccines available for man, very few. And those few the dosage has been established from numerous trials.

LIZ RICHTER: I think you're right in that establishing immune memory is one of the goals for Hepatitis B vaccine, and as Dr. Koprowski said, the dosing is very important, and different regimens will have to be tried, but I know that if I was vaccinating and I knew that my antibody titers went up and then just dropped, I would wonder whether or not I had established any memory cells.

And so I think studies need to be done, and it would be nice to see a longer -- maybe even a higher immune response from that primary feeding, and then we might feel more confident that we have established memory.

PAT SHEWEN: Pat Shewen, Ontario Veterinary College. I'm wondering when you have looked at mucosal immune responses in the intestinal tract whether you've looked for or detected IGE response and if stimulation of IGE is a consideration for safety.

LIZ RICHTER: I don't think any of our studies have looked for IGE response at this point.

PAT SHEWEN: Do you think you should?

HILARY KOPROWSKI: IGE or IgA?

PAT SHEWEN: E.

HILARY KOPROWSKI: We don't know. We know about IgA responses, and they are measured, and they are there.

PAT SHEWEN: The reason I'm asking is because food allergies are not uncommon and oftentimes IGE mediated, and the stimulus could be across the intestinal tract. Thanks.

CAROLYN DEAL: Are there any other questions for our speakers this afternoon? Yeah.

YASMIN THANAVALA: I'd like to comment on the data, if I may, that Liz showed since it came out of my lab. Just to put that in perspective for the Hepatitis studies, these mice were fed, as Liz said, a total of 16 micrograms of HBsAg across three feedings, and the peak titer in that particular construct came to about 73 MILLI IUs of antibody for ml serum.

Now, if you vaccinated with a Hepatitis B parental yeast-derived vaccine three times, 10 or 20 micrograms each time, and you make more than 10 MILLI IU per ml of antibodies, you would be successfully vaccinated. So that's just to put it in the context of the human situation.

And when, of course, these animals are challenged with a subimmunogenic dose of recombinant antigen, they make great gobs of antibodies. So it's short-lived, yes, because it's 12 weeks, but still, it's seroprotective titer.

CAROLYN DEAL: Thank you very much for that addition. In fact, that was one of the things, I think, from Dr. Koprowski's lettuce experiments.

One of the things that's always been difficult to see in the delivery of some oral vaccines is actually a good serum antibody response, and I was wondering if you could comment if you see that consistency with this delivery

system, and do you think plants have an advantage for generating a serum antibody response via the oral route?

HILARY KOPROWSKI: Well, you ask a difficult question, and the situation is complicated because we have not measured sufficiently seroresponse. That's why I hedge to tell you that the question about that.

We measure antibody. We believe that antibody has a role. I frankly today, even today more than before, am challenging everything can be explained by antibodies as protection.

So this whole subject will have to be re perhaps studied and measured now in the Hepatitis case, the T and B cells response, and this will all give you a picture of what is important.

As far as in the case of Hepatitis B, in the all volunteers in the second trial responses, and again, the question is, is it a prime sufficient for boost, or is it sufficient for protection? That's a question which we ask ourselves, and we'll have to find out.

CAROLYN DEAL: Thank you very much. Well, there's no further questions. I'd like to thank both of our speakers. I think we've seen a wonderful example of the new technology.

I think we have a break here for a half an hour. There's the posters in the back and some coffee, and we'll resume at 3 o'clock.

(Short recess.)

JIM WHITE: My name is Jim White, and work for U.S. Department of Agriculture, Plant Protection and Origin Unit in Riverdale, which is a suburb of Washington.

And it's quite an honor for me to be here, to work with this working group of people from Food and Drug Administration and our senior group at Center for Veterinary Biologics.

I've worked at APHIS since 1987 when the first field testing of transgenic plants occurred. And a few years later, there were movement ships to ship interstate or import, you know, plant-derived biologics, and from that about a decade of doing this stuff, this idea deriving of biologics from plants was very interesting, and it's obvious we've come a long way. And it's quite an honor for me to be involved in it in the small way that I am involved in it.

This afternoon's session is going to talk about environmental issues. And as you've seen some pictures from our preceding speakers for field tests with transgenic viruses or transgenic plants that these products are likely to be grown in a field.

And that will be obviously something novel for biologics. And I'm sure everybody here has heard that in the last several years batik foods and genetic engineering is a very hot issue worldwide and in Washington.

About a month ago we had a meeting with FDA, and one of the people there said that, you know, at that time the commissioners' No. 1 and 2 priority was gene therapy experiments in biotech foods.

And I know Secretary Glickman, one of his five top concerns is biotech and genetic engineering. So it's been an interesting time in Washington.

We have three speakers this afternoon. And our first who's going to speak is Michael Hansen. Michael got his Ph.D. in ecology at the University of Michigan. He's been at the Consumers Union for the past 12 years.

He's their point person on biotech issues and has written a lot about biotechnology at Consumers Union.

Michael.

MICHAEL HANSEN: Thank you very much, Jim. I'm honored to be here. I also think that I'm sort of out of place here for two reasons: No. 1, I think I'm a token critic of genetic engineering, but more importantly, No. 2, I'll be the first to admit that I'm not real knowledgeable on some of the technical details about biologics produced in plants either as transgenics or being engineered into viruses. This is sort of – and then those viruses put into plants.

This is a new area for me, and actually, I didn't even realize until last week, because I've been doing a lot of traveling internationally, that I was even supposed to speak.

So what I thought I'd do is just run through some of the concerns that I think consumers have about genetic engineering, and most of this actually refers to the first wave of products that are out there.

I'll try to do that quickly and then at the end just give some of my impressions for what I think some of the concerns about biologics in plants would be from an ecological concern because I would say one of the issues

that's of importance, I think, to consumers is the whole food safety issue.

And it is true that if you're dealing with biologics and plants, they're going to be treated like drugs, and that's a very far stricter regulatory climate. And I think the concern wouldn't necessarily be so much of the safety of some of the biologics coming out because we do have this rigorous drug review, and I think that's why you'll also see that among the critics of genetic engineering, not many of us actually talk about medical applications because while the general impression might be that all critics of genetic engineering are anti-technology or anti-genetic engineering, it's not the case.

I actually think that there are many potentially positive uses of genetic engineering, even in agriculture. The whole use of restriction fragment length polymorphisms or the use of marker-assisted selection in breeding process or even some of the development of diagnostic kits for detecting animal and plant diseases are all very positive, useful applications.

The concerns that I have and I think many consumers have as well and consumer organizations all over the world have actually deals with the food processing and the human health and environmental impacts of these engineered crops.

And I'll just very quickly say that the basic concern that I guess we have is with the regulatory structure in the United States. We don't think it's sufficient to adequately review these crops.

I will just take the Food and Drug Administration as an example. The Food and Drug Administration has argued -- well, they put out a proposed policy on how to deal with transgenic plants in May of 1992.

They have still never finalized that policy and have actually gotten -- back in '92 they got about 4,000 comments, and in this recent set of hearings that they held in three cities in November and December in the U.S. and then they also were asking for comments at the docket, and as of January 13, which was the final day, the comments were supposed to come in. The agency now tells us that they have 35,000 comments that they're actually going through.

So the basic concern I think consumers have about the regulation in the food safety area is that it's not really designed to ensure safety.

If you look at it, that policy was released on May 27, 1992, to a biotechnology. It was announced at a

biotechnology industry meeting that then-Vice President Dan Quayle introduced it as a deregulatory initiative. Okay?

So it's been introduced as a deregulatory initiative, and then years later they're trying to tell us that it somehow is going to protect consumer health.

And I think the basic concern is that the Food and Drug Administration policy basically says that genetic engineering is very -- is not really that different from conventional breeding. It's just an extension of conventional breeding. Therefore, we don't need any new regulations to deal with this. We can use existing regulations.

And I think that's where most of the problems that people have stem from, was that this regulatory framework, both at the Food and Drug Administration and then more broadly by all three agencies, the USDA, the FDA, and EPA, the so-called coordinated framework, rather than realize that genetic engineering raises questions and raises risk questions that are different than you get from conventional breeding -- rather than realize that those risks are different than you get from normal pesticides or conventionally bred plants and trying to come up with regulations to deal with that, what they've done instead is try to pigeonhole things in various places.

So the USDA, they regulate under the -- in part under the Plant Pest Act with the notion being that, well, since some of these plants are using bits and pieces of either the cauliflower mosaic virus, the promotor, or bits and pieces of Agrobacterium tumefaciens, both of those are plant pests, so there might be a probability that the plant itself could be a plant pest.

In the EPA we have the strangeness that transgenic plants are organisms regulated under TSCA, the Toxic Substance Control Act, the rationale there being that, well, these engineers, these new bacteria, such as RISOBIUM, what they really have is they have foreign genetic material in there. DNA is just a chemical, so this is like putting a new chemical out in the environment, so therefore, we use TCSA, the Toxic Substance Control Act.

And that's, in my mind, not very appropriate because the kind of safety tests you ask about a chemical are going to be different than the potential questions you ask about a living organism that's released and that can spread and move in the environment and exchange genetic material.

We then also have among the strangest categories is fish, for example. There's a lot of work being done now on transgenic fish to make them grow faster, and if you look at

the regulatory climate, there really is no appropriate regulation out there in the federal government.

So what they've come up with is the Food and Drug Administration is arguing that genetically engineered fish are new animal drugs and need to go through the new animal drug provisions, which we think is sort of curious how you can define a fish as a drug, but the agency scientists decided or the agency lawyers decided to take that so that they could make sure that all the organisms out there would be covered under some existing statute.

So I think we have this hodgepodge that if I go back to the Food and Drug Administration for a moment, I told you that their policy is one of treating genetically engineered plants no different than ones that are treated from -- ones that arise from a conventional breeding, which means that there is no mandatory requirement for safety tests. Okay? Or for labeling or even premarket notification.

Now, it is true at this point that so far all the products that are on the market, the industry has come forward and notified the agency and have done what they call the safety considerations, but there have been no real requirements except for the Flavor Saver Tomato and the CAL GENE AF that's tantamount to resistant market gene be treated as a new food additive.

So we have the FDA that basically is not requiring anything. It should be noticed that in 1994 after -- because these 4,000 comments that the agency got, actually, many of them were highly critical of the agency, and the three things that they were asking for that many of us ask for, including the state attorney generals from nine states, were premarket notification, mandatory premarket notification, premarket safety testing, and labeling.

And the agency never really responded. It should be pointed out that in 1994 the FDA actually drafted a proposed premarket notification rule, and in fact, Dr. Kessler at the time when he was being interviewed for the head of the Food and Drug Administration, he agreed that there needed to be some form of premarket notification.

They actually drafted a rule. It went to OMB. And then what happened was the elections in '94. The Republicans came to power, and functionally, that premarket notification rule disappeared.

So at the very basic level, I think people are arguing that engineered foods are different than conventional, that genetic engineering is fundamentally different than conventional breeding for a number of technical reasons which we can get into later and that there should be

mandatory requirements on all three agencies but particularly under the Food and Drug Administration.

And I'll just say we're not part of it, but in May of 1998 there was a coalition of groups sued the Food and Drug Administration saying that they're not following the federal Food Drug and Cosmetic Act, and they're asking FDA to remove all genetically engineered plants from the market until they've been adequately safety tested. And the argument is that they should go through the food additive process, and then anything that can go through that should be labeled. So we have a lawsuit there.

If we look on the Environmental Protection Agency side, there's actually a lawsuit that's in progress right now on the Bt crops that is brought folks and then a coalition of other groups.

Again, I should say in both of these lawsuits, Consumers Union is not part of either of them because we do not associate ourselves with anything that involves -- well, we do not sign onto petitions or other things that involve any for-profit entity, even if we agree with their positions. Because the appearance of conflict of interest, Consumers Union has very strict regulations about that.

So there is this Environmental Protection Agency. There is a lawsuit against them being brought by organic and other farmers because the concern there is over the impacts of Bt.

The concerns organic farmers have is that that resistance will evolve to the Bt. When that happens, since organic farmers can't use transgenic plants, that the spray applications of Bt will become useless, thereby destroying a useful pesticide for organic farmers.

It should also be pointed out that there's economic impacts now. Now that a number of countries throughout the world are either demanding labeling or segregation, the U.S. has lost about $200 million a year in export markets of corn to the European Union, and it's because of RISING corn, which were approved here that are not approved over there.

So we're starting to see the market have an effect. There was already an organic company, the TERRA PRIMA that had to destroy 180,000 bags of organic corn chips that were going into Europe because testing on the European side detected the presence of the Bt endotoxin which -- well, I should say detected that they were genetically engineered.

I think what they actually looked for was the CMV promotor. But they were able to demonstrate contamination in those organic products, and so those were destroyed.

So I think you have to -- from the viewpoint of farmers or people that are trying to sell into either the organic market or the non-GE market, pollen drift is a serious consideration.

The TERRA PRIMA, we've talked to those folks. The reason that they're not bringing -- from what I understand, I should say, the reason that a lawsuit isn't pending is because it's unclear whether the problem was contamination of drift from a neighbor's fields, or there's some concern that the seed itself at a very low level might be contaminated as well.

When I spoke in December at the National Sweet Corn Breeders Association meetings, they told me that -- representatives of one of the seed companies told me that actually that they were having problems that there was evidence that some of the sweet corn varieties that are being grown for seeds are being contaminated as well at a very low level, and this raises economic considerations, if nothing else.

So I think if we want to look at further environmental impacts, I think pollen flow is a very serious one on an economic basis.

On an ecological basis, there's the problem of movement if it's a -- for example, the Bt endotoxin, it's going to be all the organisms that eat the plant, and then the natural enemies that feed on them are going to be being exposed.

We now know that at least in the laboratory, the endotoxin appears to be being exuded from the roots. There's studies in the literature which demonstrate that there can be effects on fungal and soil communities so that they'll often see -- Kelly Donnigan and her partners have actually demonstrated from the crops that are out there, they've been able to demonstrate changes in the soil microflora.

Now, it is true that they haven't found changes at the next trophic level, but they are finding some effects which we think should be looked at further.

If we go to the issue of these producing drugs in plants, it seems to me that you have more problematic questions of environmental issues of gene flow that's either vertical or horizontal, that you have that with transgenic plants.

Now, hearing this morning and reading some of the literature that was sent to me before the meeting, it seems

to me a very interesting idea of the use of these transgenic viruses and infecting plants with them so that you don't have to worry about -- because of the instability of the transgene, it's lost very quickly so that you don't have to necessarily worry about movement of or spread of those traits via pollen flow, although from my discussions with virologists, they tell me that so little is known in this area that they wonder themselves whether they can accurately do the appropriate environmental and safety tests on this, although I should say that from what I've heard this morning and what I've read, it seems to me that you will have fewer both environmental problems and potentially fewer public consideration problems because -- maybe I'm out of place here, but it seems to me that the general public really has very little idea that the plants are being field-tested that contain pharmaceutical products in them.

We don't know which drugs they are, of course, because that's confidential business information, and I actually also know the test that's going on with some animals that are engineered to produce pharmaceutical products as well or actually to produce human proteins.

I think those are potentially – from an ethical level you might find that the general public might be very upset about those, and the reason you're not hearing anything now is that people don't really know this is happening.

How big an outcry there will be, I don't know, but it seems to me that the appropriate way to go here is to require and to be very open about the whole range of environmental and safety testing that is being done.

Again, I'm not speaking so much on the human health side because I'm assuming that since these are biologics, they're going through the drug approval process, which as I said is a very strict process, and that's why you haven't seen much concern or outcry over many of the genetic and engineered drugs that are out on the market because those are contained use, and actually, there's a very strong benefit.

But again, it's just my impression. I'll have to read more and more of this literature to sort of try to come up to speed on it, but it does seem to me that there is a potential range of ecological problems that have to be dealt with and that in general -- this is again off the cuff -- it seems to me that using the transgenic virus approach and applying that onto plants is inherently -- appears to be less problematic than engineering the plants themselves. And I also think there might be some kind of public outcry when they hear about this going on.

It seems to me the way to deal with both of these issues is for the agencies to be far more up-front with what tests they are requiring.

And on the USDA side, I'll say since I'm also on Glickman's advisory committee on ag biotechnology, there is a standing committee of the National Academy of Sciences which has been set up. They will do an investigation of how well the USDA regulations are being implemented vis-a-vis biotechnology or genetically engineered plants.

I will probably be on one of the small working groups that is coming out of this ACAB, and we will be sending technical questions over to that safety -- I'm sorry -- over to that NAS committee so that we can ensure that specific questions about the USDA's -- what they've approved and the data that they use to approve that. The NAS should be able to look at that in detail and tell us how good a job the USDA is doing. I'm somewhat confident that that committee will do a good job, and we'll have to wait and see what they come up with.

But again, my final take-home message is you need to be open and as transparent as possible because there could be some kind of public backlash when people realize that drugs are being produced in plants, that pharmaceutical drugs are being produced in plants, and might be being planted out on fields someplace because I think some of the concern would be, how much can we really regulate what farmers do in their fields? How can you really ensure that you won't see environmental impacts? And this is, again, just transgenic plants that are producing pharmaceuticals.

And I'll end by saying that the other thing that I think ecologists are concerned about is there's a range of ecological impacts that cannot be seen in small-scale field tests, that you can only see when you get to a larger scale, and that's another thing that's concerned us, that there doesn't appear to be any intermediate. You either have this small field-testing process or you have commercialization.

And I think the Ecological Society of America actually in 1989 did a fairly good report on what the ecological concerns are and pointing out that there are these scale issues. There's certain ecological effects that you'll only see when you get to larger acreage, and those cannot be predicted from smaller small-scale field trials.

So we need to have some way of having an intermediate step between small field tests and commercialization so that we can require and we can look at environmental data from this medium sized -- from this sort of semi-commercialized status.

And that's something I think needs to be thought about. It was not thought about or dealt with adequately by this first wave of products, but I think that probably the plant-producing pharmaceuticals, there will be far stricter controls placed on this, and I think even among the industry, because you are dealing with drugs and other biologically active compounds, and so you're going to be far more careful about how those are deployed than I think some of the first wave of products.

And I guess I'll end there and be available for questions later.

JIM WHITE: Thanks, Michael. The good news is USDA hasn't been sued like FDA or EPA or Biotech. The bad news is the national academy is going to investigate us, so that means I guess we're going to be answering lots of questions in the next few years.

Michael, I think, did a good job at raising two issues that our next two speakers are going to be talking about. Our next speaker, John Hammond, is going to be talking about gene flow and the movement of pollen from plants. You know, plants have sex, and you know, they don't have safe sex either. They have it, and I guess it's good that they have it since we have food from that.

John is a plant virologist. He now currently works at ARS in Beltsville where I worked before I joined APHIS. John has written a review article on risk of genetically engineered viruses. It's enhances the virus research. Maybe Michael will be interested in reading that.

And John is going to talk about shed and spread of transgenes, both from the plant point of view with pollen and from engineered viruses.

So John.

JOHN HAMMOND: Thank you, Jim. So as Jim said, I have a background in looking at resistance to plant viruses in transgenic plants and looking at the risks that some people have posited would result from deployment of those, including recombination and complementation effects.

I've also expressed an epitope from HIV on the surface of an engineered virus coat protein expressed in transgenic plants and bacteria, so this gives me some kind of background to talk about what I'm going to deal with today, which is the shed and spread of transgenes.

So I'm going to talk about the relative advantages of transgenic plants and plant viral vectors, talk about spatial gene transfer and temporal gene transfer; in other

words, spread out of the field within a season and spread in place over seasons, over time.

About the appropriate choices of crop plants for different purposes and where in the crop plant one should best express the gene, both in terms of the type of expression you want to get, the type of product it is, and the issues of containment that are concerned with each.

One of the questions under consideration is whether you should use food plants or nonfood plants. Obviously, if you're using edible vaccines, you want to express it in a food plant and probably also in cases where we process tissue, especially vegetable or fruit where a dosage needs to be established.

If the product is going to be extracted, it could be done in a food plant, or it could equally well be done in a nonfood plant so that there would not be any issues involved in the food chain with pharmaceutical products. But you have to remember that food plants do have a long history of use. That's why they're food.

Another is the question of whether it's possible to effectively use phenotypic markers to distinguish plants expressing pharmaceuticals and for purposes of monitoring any escape.

And I think that this would be useful where it's possible, so if you're starting up something, you might want to consider using visual markers. However, I don't think it's necessary. I think there are other ways of achieving these aims.

Possible markers one could use, color markers such as the anthocyanin which is expressed, especially in some lines of juvenile corn and pigments that are expressed in some varieties of fruit.

And for example, there are some very red peaches and whereas others have a much paler color. So you should choose an unusual colored marker in your appropriate crop.

Then there are many morphological markers such as ligules, stipules, glandular hairs, and shrunken seed. Shrunken seed, especially in maize it's not used so much in the commercial varieties. This might be a good marker.

Then herbicide resistance, which is better for many reasons than using antibiotic resistance as long as there are legally labeled alternatives available to eradicate volunteer plants. In other words, if you use a selectable herbicide marker to select your plants, in order to be able to eradicate volunteer plants appearing in the field, you

have to have available another herbicide that is legally registered and labeled for use on that crop.

One question that really would be useful is if you could use auxotrophs, plants that can only grow with the supply of a particular limiting substance or nutrient. In the absence of this substance, they're of low fitness. They can't grow, certainly can't reach maturity and reproduce.

Now, there is an orange pericarp mutant in corn that requires the addition of tryptophan. This can be done in hydroponic culture in the greenhouse, but I don't think this is practical in the field, neither practical nor economical.

And I'm not aware of any crop auxotrophs that could be used on a field scale economically. It's possible, however, that somebody may be able to engineer a plant so that it becomes an auxotroph with a substrate that is economically feasible for addition in the field and does have a functional auxotroph.

Question of whether you express your protein in the seed or storage organs, vegetative organs such as tubers, or whether you do it in just vegetative tissue or leaf material which is nonreproductive.

For seed you can use, as has been demonstrated, has been talked about earlier today, field materials that can be stored dry and processed at your leisure. It can be stored and used months or potentially even years later without protein deterioration. Similar issues with tubers. You can ship them and store prior them to harvest, prior to extraction.

Leaf tissue with the exception of mega-promotor system that Carole Cramer has talked about must usually be processed immediately after harvest.

And this has to be thought about on a case-by-case basis, whether you need to contain on site versus your requirements for storage and processing later, and that's going to depend on what you're doing.

Another question is whether the plant in which you are expressing your gene has weedy relatives. Rapeseed does have weedy relatives and has propensity to interbreed with them, but there is a well-developed system using the oil-body protein, protein that secreted into the seed oil bodies and for efficient extraction and separation of these materials later. And that's a significant advantage that should not be overlooked. So one has to deal with this and work out the appropriate containments conditions.

Rapeseed is unlikely that it would be approved for large-scale usage because on a large scale, it's much more difficult to effectively use containment. There are much larger populations of weeds that you would need to control and eradicate, and that's much more difficult to do on a large scale.

But on a small scale within a geographic area in which there are not related crops and all you have to worry about are the weeds, I don't see why one should not use rapeseed. But the question of scale is one that must be examined. There are differences between large and small populations in large areas.

Okay. Coming back to the differences between transgenic plants and viral vectors, and Dr. Koprowski talked about some of these earlier. Transgenic plants have stable expression over multiple generations. You have the possibility of using promotors that are specific for particular tissues or organs, are expressed at particular times during development, or can be induced by addition of a chemical compound to turn the expression on. Transgenic plants take relatively longer to produce and longer to scale up than do plant viral vectors.

Plant viral vectors have the advantage of biological containment that over a period of time and especially as you transfer from one plant sequentially through other generations of plants, tend to lose the inserted gene, so that's an effective biological containment method.

It can be done very fast. We've heard this morning and from Large Scale Biology that they can produce things in a matter of 10 days and scale it up extremely quickly to field scale.

But these are expressed primarily in foliar tissue. There are some viruses that are spread more specifically in other tissues, but by and large, viruses are expressed in foliar tissue.

Definitely if you're going to use plant viral vectors, you want to choose nonvectored viruses or isolates. Even for many of the viruses that are normally vectored by aphids or nematodes or white flies, there are nontransmissible isolates available. You can take an isolate and engineer it to knock out the vectored capacity, and certainly one would want to do this. And one would also want to avoid seed-transmitted viruses as this could have the potential for escape through time.

The other possibility that Dr. Koprowski mentioned this morning was the use of transgenic host plants that complement a defective viral vector so that you can only

have replication of the virus in that transgenic host, and therefore, it's very limited in space and time to where you can grow the product.

And then there are viruses available that have different host ranges, and it would probably be preferable to use one that has a narrow host range rather than a broad host range.

You can grow the crop that the virus infects and avoid having it near crops that it would otherwise infect, so you can limit it by growing a crop surrounded by unrelated crops.

Most viral vectors, I already mentioned, lack long-term stability. The introduced gene is deleted, and typically, the resulting deletion is essential wild-type.

Most modified viruses are somewhat debilitated. They don't replicate quite as effectively as the wild-type virus, so when you do get the wild-type virus produced by the elimination of the insert, the wild-type virus will outcompete the modified virus.

And using a nonsusceptible crop as a subsequent planting will limit severely the possibility that the virus can continue to exist in that place and, again, avoidance of seed-transmitted viruses.

Weed control will minimize the availability of ultimate hosts. Large Scale Biology talked about this, and Jim mentioned how few weeds were found in those fields and how little they were able to find evidence of transmission within the field.

In order to monitor, you can test using ELISA, which is a serological procedure, or PCR or bioassay of any symptomatic plants or selecting randomly from nonsymptomatic plants to determine whether your virus is escaping into the weed plants growing in the field.

Washing, bleach treating, or otherwise inactivating virus on farm equipment that is used in the field before that equipment is taken anywhere else will limit the spread. BioSource, again, talked about that.

And then destruction of field residues by disking it into the ground. When this material is composted, desiccated, or herbicide treated to burn it, the virus will likewise lose viability. Viruses can only retain infectivity in whole cells on surfaces for a very limited time. UV light will inactivate it quite quickly.

And planting a nonsusceptible crop, especially after a fallow period with weed destruction will almost guarantee that there's not any virus carryover to subsequent crops or years.

Now, moving back to transgenic plants, limiting gene transfer to sexually compatible plants. One option is using highly self-compatible plants that inbreed almost exclusively.

You can use plants with male sterility mechanisms, crops without noncultivated relatives, crops that don't readily overwinter. There's not much in the way of volunteer plants from many crops because the seeds under most conditions will not overwinter. We have to harvest the seed and replant them the following year, in part because they don't overwinter.

Apomixis. This is a state in which a plant will produce seed without having sex. The seed is produced by and is identical to the maternal plant, and in this case there's no gene flows, only from that seed that is produced. So if you harvest that or clean that up, kill any volunteer plants, you're essentially eliminating any gene flow.

Chloroplast transformation has been touted as a mean of containment. It's not complete containment by any means, but in general the chloroplast is transmitted maternally, and this does predominate in most species, although in many species there is some paternal transfer of chloroplast genomes as well. The chloroplast transformation has some other advantages that I'll come back to later.

And then this terminator technology. Now, terminator technology has gotten an awful lot of bad press from an awful lot of different sides, and some of that is justified.

But for the containment of pharmaceutical products, this is exactly the kind of application that terminator technology was designed for, to prevent gene flow. And this is an ideal case for its application.

Okay. Highly self-compatible inbreeding crops. Soybean almost exclusively inbreeds. The pollen fertilizes the same flower. Foundation seed requirements are zero separation because of this habit. The anthers mature in the bud and directly coat the stigma of the same flower.

Cross-pollination is less than 1 percent, and in many lines it's much less than 1 percent. It's barely detectable. The breeding lines are pure breeding homozygous lines, and cross-breeding, cross-pollination, manual cross-pollination is required in order to breed new soybeans.

There are no sexually compatible weed species or other crop species present in the United States except for those other species that are deliberately grown at breeding stations for this purpose. You're not going to find that out in the field. And there's a very low rate of seed-producing volunteer plants in subsequent seasons.

Now, in corn there are several male sterility mechanisms known. The best of these is the Texas cytoplasm, which is susceptible to Southern corn leaf blight, so I wouldn't suggest the use of this plant seed for production because of the problem of loss of yield and also the potential contamination from the fungus.

But there are two other types of cms: cms-C and cms-S, which are resistant to the virulent race T of Southern corn leaf blight but have less than complete sterility.

Now, male sterility only blocks gene flow out of the crop. It doesn't block pollen flow into the crop producing a hybrid seed that can drop and remain in the crop. So there's still a need to look out for volunteers and destroy them, but this does prevent, I guess, contamination of other crops is the word that some people like to use.

Now, there's also a nuclear male sterility in corn. This is recessive, and you're unable to maintain pure male sterile lines. So this is of rather limited usage, so it wouldn't be a useful containment system in this case. There are similar male sterility systems in several other crops.

Okay. There are several crops that don't have any wild or weedy relatives within the United States. Corn is one example of this. Teosinte, which is the closest relative of corn, is only found in Central and South America. It does not occur in the United States. And corn is incapable of sustained reproduction outside of cultivation.

You may see a few volunteer plants, but if those grow to produce seed, it's unlikely that you will have -- you don't find naturalized populations of corn anywhere.

Similarly with soybeans. The related species are not native to the United States. It grows essentially as an annual plant, and there's little shed seed that germinates the next season.

Apomict, those crops that can produce seed without fertilization. There are a number of crops that do produce fairly readily seed without fertilization. Citrus is one case in point. Meadowgrass is another. Sorghum can produce seed apomictically but it is also readily outcrossed.

There aren't many major field crops that are stable apomicts, although there is the possibility of using genetic engineering or conventional breeding techniques to increase the degree of apomixis and thus limit the degree of outcrossing that is possible.

This has potential use to fix heterosis in hybrids, so you can have essentially a line of permanent F1 hybrids and not have to recreate your seed every year.

And apomixis most commonly arises from breeding programs with wide hybrids and polyploids and perennial species rather than annual species.

Now, there are some crops that are produced primarily by vegetative propagation. Potato is one example of this. Potato can be produced by true seed, but in most seasons there are relatively few fertile true seed produced in this country in this environment.

It is an allopolyploid genome, so the progeny from such seeds in most cases differ from the parental type and are easy to spot. However, when you're dealing with a crop that is produced vegetatively, volunteer plants are common.

If you grow a potato crop and you go back to the field the next year, you will find a lot of potato plants coming up in between whatever the other crop is. And so it would be necessary using a vegetatively propagated crop to go through and take care of these volunteers, either mechanically rougueing them out or using herbicides to eliminate them.

Sugarcane and banana are other examples of crops that are produced vegetatively; sugarcane from stem pieces and banana from trees. And banana is almost totally sterile, which makes it one of the most difficult crops to breed.

Chloroplast transformation. It's possible to get high copy number without so much of the silencing that occurs in other systems, and thus, you can lead to very high expression levels; also, because there are many copies of the chloroplast within the individual cell.

Maternal inheritance is the primary means of inheritance in most crops. However, especially among gymnosperms and some genera of angiosperms, paternal inheritance is also quite common, and it doesn't prevent gene escape because there can still be pollination of the transgenic plant. And then you have the chloroplast genome from the transgenic plant serving as the parent and providing the chloroplast to the next generation. So it's only partial containment, but it can be used in conjunction with some of the other methods of containment.

As I mentioned before, pharmaceutical-expressing plants are an excellent application for terminator technology. It prevents gene flow by either the pollen or seed, at least beyond the next generation, and it's entirely appropriate usage to prevent unintended expression or volunteer plants.

Physical means of limiting gene transfer. With corn plants you can emasculate the plants readily by going through and detasseling, thus preventing pollen flow out of your transgenic block.

You can physically isolate your transgenic plants, and the distance necessary depends on whether they're wind pollinated, insect pollinated, or self-fertile.

You can do this by planting barrier rows of nontransgenic plants to trap the pollen around the block and then either destroying those or harvesting those and using them for some other purpose and by using barren strips of land between your transgenic plot and surrounding crops just to provide physical distance, and that depends on the pollination mechanism.

And if possible, depending on where in the transgenic plant you are expressing the gene of interest, you can harvest plants prior to flowering or seed maturity and thus prevent the possibility of volunteers being available through seed for the next season.

Okay. Corn is wind pollinated and, as a result, has a relatively high distance that's required for separation of foundation seed, about 660 feet. The standard for pharmaceutical-expressing crops is double that, 1,320 feet.

Now, depending upon the size of the block of plants, the dilution effect is greater or lesser. If you have a relatively small number of transgenic plants, the effective distance which that pollen can travel and the effective number of weeds within that radius that could be pollinated is different from if you have a very large block and a small number of nontransgenic plants which could be pollinated. Those effects differ on whether you have a large block and a large block or a large block and a small block or a small block and a large block. And that's been discussed by Peter Kareiva and Norm Elstrand. I can't go into that in any more detail.

For rice the distance required for foundation seed is 10 feet, and pharmaceutical requirement would be 20 feet. Soybean, there's zero space required for production of transgenic seed, and what would be required for pharmaceutical production would be barren-space separating

plots. So depending upon the type of crop you're looking at, the type of isolation, distance of isolation can be quite different.

To limit transgene persistence in situ. If you're expressing in vegetative tissues, you can harvest prior to flowering and destroy the crop residues, and that will essentially take care of things. You obviously need to go through and make sure that there is no material surviving the destruction using herbicide or mechanical means to destroy the planting.

For expression in seed or vegetative storage organs, it would be necessary following harvest to leave the land fallow, treating it by irrigation if necessary to encourage germination or sprouting, and then destroying all the volunteer plants that come up.

Following in all cases by replanting with a clearly visibly distinguishable crop and screening for and eliminating any volunteer plants. And as I mentioned before, if you're going to use a herbicide resistance for transgenic selection, you must have a legal alternative available for destruction of any volunteer plants.

Okay. Within corn, corn is wind pollinated, and it's capable of both self and cross-pollination, depending on the spatial relationship between blocks and other physical factors.

The pollen is typically only viable for 10 to 30 minutes from the time it's shed, and that's one of the factors that limits the distance that effective pollination can occur over. And the likelihood of pollination decreases with distance, both because the pollen is more likely to drop to the ground and because there are less likely to be plants within that range, and the pollen viability will drop.

Detasseling of your corn will prevent gene flow out of the block but not gene flow from the outside in. So you won't have transgene moving out, but you could have seed being produced on your transgenic plants that contain genes from other sources.

There's no vegetative reproduction of corn. It occurs only via seed, and seed rarely persists as volunteer plants. And there are no sexually compatible species that grow in the U.S.

So these are some of the things that you can look at, and there are similar considerations with -- or different considerations depending on the crop. With soybean, which is highly self-fertile and essentially impossible to

outcross, unless you are doing it manually, the considerations are very different.

So what type of host plant and what type of containment should you be using for your transgenic plant-expressing pharmaceuticals? Everything is going to be case-by-case determined depending on the type of product and the degree of purification that is required from your crop.

Seed expression has many advantages for storage prior to extraction of the product because seed is stable under a range of temperature and humidity conditions and can be stable for long periods of time, months or even years, depending upon the type of seed that you're dealing with.

For leaf or organ expression, it is generally required to extract from fresh material, and in one should harvest prior to either flowering or seeding. And then the scale in which you are producing the transgenic crop influences significantly the type of containment that's necessary.

I mentioned rapeseed earlier. There are approved trials of rapeseed in Canada in an isolated inter-mountain valley, well away from commercial crops and on a relatively small scale, and under these conditions it should be quite feasible to contain any weed hybridization that occurs. This would not be possible on a large scale, and thus, this crop would not be appropriate for growing on a large scale for pharmaceutical purposes.

But all of these factors need to be dealt with case by case. It's very hard to set up hard-and-fast rules by which you can say this will work because it will work with one crop, and it won't work in another crop.

So all of these things need to be considered with an individual product and an individual host plant that you're growing and for viruses as well.

(Off-the-record discussion.)

JIM WHITE: Our next speaker is Charles Rupprecht for weed control. I think it's very appropriate that he's here. As John has talked about field-testing, one of the considerations for regulatory agencies is to be compliant with the National Environmental Policy Act, NEPA, so we have to consider impacts on non-target organisms. And obviously, BioSource mentioned there are deer and rabbits and squirrels and all those warm and fuzzy animals that are out there, and if we have large scale growing of plant biologics, that's an issue the regulatory agencies will have to address.

And Dr. Rupprecht was involved when I came to APHIS and a former organization -- I was in Biotechnology, Biologics, and Environmental Protection with Dave Espeseth who's going to lead this public hearing, and Dr. Rupprecht was involved in looking at non-target effects for the Pseudorabies virus and on wildlife.

And so he's going to talk about the research that has been done based on animal vaccines on non-target effects on wildlife and comment on the potential for using plant-derived biologics.

CHARLES RUPPRECHT: Not to correct my elders, but it was for rabies virus, not Pseudorabies virus.

If I could have the first slide, please. I would like to thank the organizers for their very kind invitation to participate in this symposium. We've been very impressed by the expertise of the speakers and their presentations. Also like to thank my coauthor for some of our musings about these issues of non-target species, and particularly because of points over those entities that could either fly, hop, crawl, or otherwise burrow into some of these experimental plots, which are not so much of a problem when these are done in containment, but certainly when they're conducted done via "less than containment", I think someone needs at least to be raising some questions.

At this point I don't think there is any preconceived notions among the scientifically informed that would certainly raise any apocalyptic notions nor cause a burning skid to a moratorium on any of this research similar to what was done in the '80s or suggested thereof when recombinant technology first came to be.

But on the other hand, I don't think that there are the majority of knowledgeable or informed sources that would tomorrow vis-à-vis want to have the ad lib plantings everywhere for consumption of non-targets without prior testing, particularly for biologics that could be viewed as pharmaceuticals for human or veterinary purposes.

There were comments about most of us would assume and agree that we've been consuming over the course of our lifetimes relatively large amounts of viruses in uncooked vegetables. I would think that also one would agree that for thousands of years, people have also been consuming fermented beverages, and some would still argue that those could not have clinical effects.

Similarly, our love for certain things from certain countries that produce the best hand-rolled cigars as to their clinical effects and some of the issues that have been going on and until one looked for that proverbial tree

falling in the forest, there's still arguments about the relative risks of things people have been doing for thousands of years.

Similarly, no one wants to rain on the parade, but in our involvement in the working group for 'xenozoonoses' from the use of xenotransplantation may suggest otherwise, and clearly there are brave new worlds that bring up whole new realms of regulatory issues, previously not considered.

Also from some discussions with our colleagues that deal with STDs, there are a variety of things that are going on and probably have for thousands of years that give new definition to the human animal bond, if you will, as to levels of intimacy that oftentimes we don't discuss in public forums, probably due to a conservative upbringing.

Thus, there are a variety of "risky" human behaviors that have undoubtedly been going on and still do, but for a variety of reasons, including limitations of technology or for even societal concerns, we haven't even questioned.

Similarly, from an epidemiological perspective, while we've been consuming plant viruses daily we have no documentation that plant biologics or plant viruses have any notable clinical effects on people or other mammals, but one has to ask the question, and to raise the issue, of how hard one has looked.

From a virological standpoint, there are many, many more unclassified viruses than classified and many, many more uncharacterized viruses than those few that we deal with, and one hopes that given the great intro that our previous speaker presented that some of those issues of using what's common and known would be supported as opposed to using the truly novel because of some of those potential repercussions.

And similarly, it would be very, very difficult to either have prospective or retrospective epidemiological studies unless those questions have ever been raised as to could plant viruses be involved in any clinical conditions, be they human or other animal?

In fact, it wouldn't be too difficult to predict that now that we've got the tools available and given the realms of the majority of uncharacterized plant viruses that sooner or later somebody will put that connection together. It just hasn't been done yet.

And so I think we have to keep some open minds because if we don't, then surely we are open to criticism for those that apparently are less knowledgeable than ourselves.

Why study wildlife at all? Well, one thing is because they can serve as surrogates to humans and also surrogates to veterinary species as to potential free choice of various biologics out there.

Moreover, wildlife are used whether they run, crawl, fly, hop, et cetera, for both food, fur, and fiber, in consumptive uses. And similarly, there are a variety of wildlife in the broadest sense that consume these biologics out there and for which we have little or no control.

Some of the previous speakers have addressed issues that some of these plants will be grown certainly with little or no containment versus for biologics, be they hepatitis, rabies, hepa-proteins, et cetera. They're only viewed as being grown under containment issues or procedures.

There are also a variety of ecological and ethical issues that one needs to raise if, in fact, we really want to be good stewards and have answers for those that raise some of these questions that maybe we haven't had enough time to pause for concern. I've thought about these in some very broad terms in terms of host agent environmental issues along the lines of that broad epidemiological pyramid that obviously it is going to depend upon what it is that one is talking about.

What is the bug, the agent, the gene, the gene product that one is talking about? Are we talking about long term as opposed to single season plants, plant for release? Are we talking about things that really can be considered under the standpoint of human or veterinary biologics?

What is our experience? Show us the data. What do we know from experience, either because as we'll characterize with TMV, it's something that one has decades of experience with as opposed to something that one has relatively little to no experience with.

There's also the issue over dose. Are we talking about exposures of picogram —amounts, or are we talking about exposures to potentially kilogram amounts of something that particularly from an endangered species standpoint could gain access to?

Similarly, what about spatiotemporal issues? How long is that product going to be available to species X, Y, or Z for which it's not intended? Is it going to be only in place, or how mobile, either from wind, water, or through inanimate means would any of those products be expected to move or stay in place?

And similarly, what does one project is the degrees of exposures between GMOs and non-target species? Non-target, again, in the broadest wildlife sense of not, what was intending for it to come in contact with.

As to the particular agent, ours was the vaccine rabies glycoprotein recombinant, and obviously, there are a whole suite of considerations that were relevant to that bug that aren't relevant by and large to most of the topic under discussion today and tomorrow.

Similarly, one has to recognize that it was in 1983 that we started first playing around with this recombinant agent, and it was a pediatrician who then expressed to me that this bug would never be allowed out of the laboratory.

As far as I'm aware, it was the first Category 3 application, and eventual field test in 1990, to USDA at the time, recognizing that pseudorabies GMO was not in the same category, as a deletion mutant. The V-RG had the incorporation of rabies glyco-protein.

And so there were some great considerations that we gave to that at the time for which there were no guidelines; i.e., there was not necessarily before I became a public servant and certainly some of my colleagues in the audience who also were, there was no mandatory government oversight of saying maximally of what you had to do because these were relatively new grounds.

Similarly, it wasn't industry since we were in academia in that sense who was saying, "This is what we really feel to be prudent." And so we struggled along that way, along the lines of the academic regulatory as well as industrial environment at that time as opposed to if it was only left to one of those entities in and of themselves. There weren't firm guidelines.

Similarly, we have to recognize that there are some viruses, hopefully none that are going to be used for vectoring purposes, that have the ability to replicate in plant or in animal tissue at least invertebrate or invertebrate and vertebrate tissues.

But again, at least to this point, I'm unaware of any that have the ability to replicate in all three or from the botanical to the vertebrate, at least not that has survived such introspection or lack of since there's not a great plethora of data out there.

We know, for instance, of even among the Rhabdoviruses, and there are other viral families that have these abilities, again, some of which we hope won't be selected for any vectoring purposes.

Similarly, when we're talking about effects upon hosts that we're not just relegated to the control of looking at the standard rodent or lagomorph model because it's convenient, things that are highly inbred and that are useful for tried and true experimental purposes that really if we are concerned about wildlife, there are a variety of wildlife out there for which we don't have to resort to just ICR or mice, et cetera.

Now, obviously, these sorts of considerations are not going to apply to the majority or even, we hope, to a wide variety of applications that are going to come into play today or last week if the horse is already out of the barn or certainly next year.

But if one has some concerns, one would hope that there is some regulatory oversight to bring up some of these issues such as which hosts are one going to deal with as far as non-target effects? Are you going to try and adapt, for example, a field mouse, a raccoon, or a deer model, for experimental insight, as similar as what's been done for lab rodents?

What about age, sex, reproductive effects? What about immunosuppression effects? There was nobody telling us that we had to look at FIV mice. We felt it was incumbent upon ourselves because as Robin Williams taught us in Aladdin, that genie doesn't like to go back into the bottle oftentimes.

And so we felt that given the potential repercussions, no matter how much science fiction, it was incumbent upon us to be good stewards and raise those issues rather than have them raised of us after the fact.

Our animal model, of course, was the raccoon as per our target species at the time and also may or may not be a relevant one; for example, with corn is one of your target plants or how one presents the introduction of those animals.

But we didn't limit it to our target species raccoon. Obviously, in the course before one even field-tested in 1990, the gamut ranged all the way from nonplacental mammals to nontraditional terrestrial mammals.

In fact, in some of our considerations early on, it ranged more than what would be likely to come in contact with this product when vaccine would be incorporated and distributed for free choice uptake by somehow.

There are also environmental issues, land use issues, that have to be taken into effect as if one does some trial

with some biologic out of the lab, where are you going to do it, and what other land uses are going along at the time?

And so it may be all in containment, or it could be in segregated plots, as we've heard quite a bit about, or depending upon the relative benefits and the relative risks of the agent in mind and the hosts in mind, it could be as extreme as to even more severe isolation as to where you want to do this, if not to prove it to yourselves then at least to prove it to society that's raising some of these questions once they find out about what it is that we're doing or hope to do.

There were also issues of scale as well as we've heard about and timing as to a single time or seasonally or ad infinitum, et cetera, and what sort of call-back potentials there are if, in fact, we find out that maybe we did something wrong.

As far as how one goes about making some of these considerations, obviously, they're going to be based upon multidisciplinary input as to things that in great likelihood we'll be consuming these products, either from direct observation or, similarly, whether one ever needs to go to the necessity of a true island environment, whether it is an actual island as we had to or whether one in 1978 that the Swiss did when the first oral biologics were released for rabies vaccination and rather isolated ecosystems or, in fact, if we feel confident enough that we can go ahead and have some of these trials take place ad hoc.

How does one go ahead and consider some potential adverse events that can occur from the introduction of these bugs, vehicles, plants, plant products over time such as potential interactions or notable observations as to adverse events?

What do we need to look for if it doesn't spring up automatically and beat us about the head, if you will. In other words, from an onion skin approach that it's almost obvious to some of us who do lay awake at night trying to do the Jack Nicholson of A Few Good Men so that others can sleep well at night, obviously, if things die or if they get sick or hopefully if one is doing necropsies, there are gross lesions or microscopic lesions or ultra-structural lesions or physiological alterations, et cetera, hopefully somebody has at least given this some forethought and has a means of beating the bushes looking for bodies that could be out there or utilization of live trapping to look for adverse events that could be related to release of Product X, Y, or Z.

Or are there any gross lesions that become intuitively obvious in your released versus nonreleased control areas

for said non-target species? Or upon necropsies of suitable statistical sample, are there any gross lesions that can be identified and similar postmortem follow-up for target organs of choice, again, depending upon what antigens, biologics, et cetera, one should concentrate upon as to organ systems.

And similarly, whether or not if there are suggestions as to some adverse events if one actually has to go to any ultra-structural levels to look for that degree of potential adverse events.

Similarly, there are things that one needs to be cognizant of that may not present necessarily as over death, say, the Calicivirus virus situation for a bug that wasn't immediately supposed to be released in Australia or any of the lesions that I've described so far. Even very, very subtle things such as, are we going to see changes in weight between control versus study areas?

And similarly, what's normal out there? Even with an animal like raccoon that you think we knew everything there was to know about, me and my colleague Dr. Hamir who now works for USDA, we still have papers on the drawing board pre-1990, and we thought just about everything there was to know about raccoons. Well, that's not the case. So one has to determine that what normal is in order to compare what abnormal is based on your facet of potential adverse events.

And beyond the biology of what it is that could be eating, hopefully you've got some other studies that are ongoing to actually document things that are coming into these plots and potentially are actually eating them.

And if, in fact, from observations of nontransgenic bugs, plants, et cetera, you get some idea of what hosts one should go ahead and compare and consider under captive conditions first.

In our situation we were embarking upon Noah's Arc with over 40 vertebrates that were tested before it was ever released into the field. At the time it was a pain, haranguing over some of these issues, and yet in retrospect, I think we sleep a little bit better that we did some of these studies in captivity before ever going into the field and thinking about issues of hosts, bugs, environments, et cetera.

Similarly, the kinds of techniques that one is going to use to survey over time, depending upon the nature of one's release and the potential effects in that community, utilization of live trapping, for example, and multiple mark recaptures, issues related to normal parameters, blood physiology, et cetera, looking for antibody effects, how one

goes about getting your sample such as roadkill effects and looking for lesions in some of those target organs, similarly looking at non-target species for histopathological lesions based on gross introspections for a wide variety of species and also the possibilities, again, depending upon circumstance for radio collaring, telemetry.

We've certainly come a long way, even from the 1990s, as to the ability to very inexpensively mark these animals and monitor them over distance and have both activity as well as mortality indices that one gets a signal that, in fact, your animal has died in a control versus experimental plots that one gets their hands on that carcass in a timely fashion.

And one could go ahead and almost looking at survivorship studied in Phase 1, Phase 2 human clinical trials, similarly, one can do these same kinds of manipulations in wildlife populations and studies of choice as well as looking at some more nebulous potential effects as to effects on biodiversity, movement patterns, et cetera, of one's small mammal of choice.

We also have to recognize that there are a whole variety of issues beyond the scientific ones that I think we have come a long way from those days in the 1980s and always having to have adversarial relationships.

I think if we can consider what some of the problems of perception are out there, we oftentimes recognize that scientists in ivory towers and gene jockeys oftentimes don't make the best spokespeople, that they want to be purists in their research, that we have to have that need for outreach, that oftentimes we have to have our data speak for itself rather than mere speculation or subjectivity, and oftentimes those data aren't out there, and if one is not transparent, then you really can't blame some members of society to raise some of these issues and oftentimes very vociferously.

There are also some issues that have already been raised about in whose backyard these things might be going on versus obviously these really are going on in everybody's backyard, so to speak, if, in fact, we can't call these back.

And on issues of scale that after some rather laborious hand-wringing and actual generation of data that there really is anything to fear that it's not tomorrow that you go from upscale of only having one or a few trials of product under question to having the millions of doses.

And so again, from along the lines of what one has been able to do with oral vaccines for rabies in this country from ideas in the '60s to products, recombinant

products, in the '80s to first field trials in the 1990s to now millions of doses being utilized in the U.S. without apparent consequence. But again, that's because people are still looking for apparent consequences from a public health or from a veterinary, both domestic and wildlife avenues.

This was certainly not a random sample, but I did solicit some opinions on these issues from colleagues, not random because these were very knowledgeable individuals with various levels of expertise in molecular virology, immunology, wildlife biology, et cetera, veterinary medicine.

Moreover, it wasn't random because they knew they could share their opinions regardless of what I was going to think. And almost to the one, they had no grave concerns over the topics of which we're under discussion today and tomorrow, but that was with the caveat of with the proper federal oversight and that by and large, they were unfamiliar with the data as to what had been done to date, either for the kinds of issues that are under discussion here for which we laud both regulatory authorities to bringing to greater light but also because of their by and large ignorance of the data that suggests that there were no adverse events in some of the populations that we're talking about; i.e., wildlife.

And so there are a variety of professional organizations that are out there, American Association of Wildlife Veterinarians, Southeastern Cooperative of Wildlife Disease Studies, the Wildlife Disease Association, and on that are nonfederal agencies whose job it actually is to go ahead and look for potential adverse events because things change. Stuff happens.

Continents who say they are free of a disease historically but never looked find out in 1996 as late that they have a brand-new bug. Stuff happens. And so one needs to have a dialogue not only with the public but with some of those professionals out there whose job it is to try and have some oversight and good stewardship.

in our little experiments with VRG over the years, it was a multidisciplinary international collaboration that went way beyond just industry. In fact, it wasn't industry that was pooling these things along. It was the consortium between academia, industry, and government. And I think that's the kind of partnership you're going to have to maintain if, in fact, you're going to be able to build the relationship with the greater public at large.

I think in conclusion, putting these things in perspective, that we need to be thinking outside the box because if we're not, who else is? We need to be doing our

homework as to how good we feel about these things. We have to be questioning our own motivations.

I question these issues whether or not based on our first speaker there ever really was going to be available for the developing world. And so if, in fact, we're talking about the kinds of orders of magnitude and put it in perspective from the rabies situation, we know parenterally somewhere down to the few microgram realm, we know parenterally can be used to vaccinate an animal once.

And that's only on the order of about 25 cents, U.S. cents. And so really, are we going to be able to come up with a biologic that on the global market en masse -- I'm thinking from a dog vaccination standpoint or any veterinary applications -- are you going to be able to compete with that?

Also, in addition, zoonoses and the question of human pharmaceuticals, by and large, we should be focusing on the reservoir or reservoirs as opposed to worst case of having to deal with humans.

But if, in fact, when things fail and humans do get exposed to zoonotic agents that live in animals, are we ever going to compete with, again, from rabies biologics as Dr. Koprowski mentioned, these things are beyond the scope of the developing world.

And so although there's certainly the promise, I really doubt because of regulatory issues and good manufacturing processes and QA, QMC if we're ever going to see the reality of being able to provide these things, I'm thinking replacements for rabies immunoglobulins, for example, which is something we grapple with all the time, I just don't think that's a reality.

And if, in fact, for some of these organs, because most zoonoses never make the Top 10 of diseases, then if some forethought should be given to the not for profit use of these or, in fact, developed world bear the brunt of higher prices in order to make these more available to developing countries.

Let's not be fooling ourselves that these things are going to be as inexpensive as water because, by and large, industry is there not to be taking a loss on these products and, at the very minimum, have to recoup their losses.

And similarly, I think it is very true that from a regulatory perspective these are all going to have to be handled on a case-by-case basis. There is no cookie cutter approach to the kinds of discussions that we have here nor I hope in the near future.

I thank you for your attention.

JIM WHITE: I'm going to open the floor for some questions for our speakers and John. I don't know where John is sitting.

Does anybody have any questions? Would you come to the microphone?

ALLEN MILLER: Yeah. This question is for John and maybe Dr. Koprowski. We talked about making transgenic plants express the viral replicase as sort of the way to control, so then you only need to put in the RNAs that get replicated so that you don't have to worry about the virus escaping and spreading.

Do you think there's a risk, though, of RNAs evolving in the plant that become templates for the replicate or incoming RNAs that may be defective that become DI RNAs and replicate, and speaking kind of about the data I believe that you expressed the replicase in the absence of any templates is just finding one, starts making RNAs, and that's not too good for the bacteria.

Have you thought of that?

JOHN HAMMOND: Certainly can't exclude the possibility that something would happen. The put in have lacked their own replication signals, so they can't get out and do anything else.

So while it's possible that something might replicate within that plant, I don't see how it would get out except in combination with another virus.

ALLEN MILLER: Yeah. It may not get out. I'm thinking the main thing is you might see a mutation on those plants. Maybe the plants that are thrown away earlier really aren't looked at.

JOHN HAMMOND: Well, that's certainly containment in situ, and if you were to observe any odd phenotype, it would be a relatively simple matter to identify that. That would certainly be one of the first things that you would look for if you found something like that.

ALLEN MILLER: But, you know, there are some logistic interventions between viruses, and I was just sort of thinking that that might help along the incoming virus as well. I don't know. It's just something to consider.

JOHN HAMMOND: There are cases where things have arisen. Satellite in cucumber mosaic, I think, is at least

presumed to arise de novo, that it may be a defective plant RNA that is captured by the virus and replicated. And I'm sure there are other instances.

I'm not sure that those could also not occur in a natural virus infection. I don't think that we would be providing the opportunity for anything in a transgenic plant that cannot occur already in a mixed infection in nature.

ALLEN MILLER: Okay.

JOHN HAMMOND: And you must admit that the prevalence of mixed infections in nature is very high and has been occurring in many species over many hundreds and thousands of years. Why should a defective transgene in a plant lead to something that hasn't happened in natural situations?

ALLEN MILLER: Okay. Well, that's just the age-old question then.

JULIAN MA: Julian Ma. I have a question of Dr. Hansen. But I'd like to preface it with just a couple of remarks.

Coming from the U.K., I'm well aware of some of the arguments that he's put forward. We have a very -- as everyone knows, we have a very negative public opinion against GM foods and crops, although I should hesitate to call it public opinion because I think the public has had nothing to do with it, and it's more of a public hysteria whipped up by the media and various public organizations.

Actually, it's the very attempt to be open by the U.K. government in putting a Website which described the locations of the larger kinds of field trials that you describe and advocated that has led to them being targeted and been closed down in the main, and I find that very depressing.

The other thing that I find depressing is the regulation of science in this whole debate. Most of the critics in the U.K. and Europe are quite eager to say that they "don't really understand the underlying science, but." And I'm also a bit depressed when you prefaced your talk with similar comments, although having heard your talk, I realize that you are probably as aware of the science as any of us are here.

So my question is, firstly, do you agree that the debate and the questions can only really be answered by application of the scientific method? And if so, how do we bring science back into the debate?

MICHAEL HANSEN: Actually, I think those are very good questions. I'll first start that -- I'll first start out by saying I think part of the problem you have in the United Kingdom is the lack of trust in the public of what government regulators say, in fact, because the 10 to 12 years when the government said with BSE that there wasn't any risk here at all. We have nothing to worry about. And then they learned in '96 and later that that wasn't true. So I think that's a special case.

But in terms of -- I do agree that we have to try to get science back into this, but I also think that there is a role to play by the public and that -- I mean John Durront at the public museum in London has done wonderful work pointing out that you have to have these consensus conferences and do things in an, you know, open and transparent fashion.

And in fact, at the OECD meeting in Edinborough, John Durront was very strong and basically said that you have to explain this stuff to the public. They have to feel like they're having some kind of input.

I actually agree. We should look at this on a scientific basis, and in part we don't see a lot of that in the general media. We see things on both sides. We see the extremist statements of people talking about apocalypse, but we also see on the other side people saying that this is the most rigorously studied and regulated, you know, plants in history, and that's just not the case.

I think folks in Europe and elsewhere actually get somewhat surprised when they hear that there is not a mandatory premarket review process in the United States for food safety.

So I say yeah. Let's open it up and actually get on the board for EPA and FDA and USDA the kinds of questions that should be being asked. And I think some of the more technical critics of us have done that in comments that we've submitted to the agency with very detailed, you know, technical considerations.

I mean I'll talk with you afterwards about this, but I agree that that's important, but we also have to allow the public to have an input because there are issues that also have to do with morality in a strange way.

I mean people in terms of how risk-averse they are, part of the consideration isn't just a technical one. There's all these value judgments that come in such as people tend to be much less willing to take a risk if they feel that it's involuntary and something that's imposed on

them with no particular benefit than if they're taking the risk voluntarily themselves.

So there's all sorts of considerations that I think are also not strictly scientific that have to be aired as well, and I think the use of these consensus conferences as has happened in the U.K. and Denmark and elsewhere would be a very good model.

And I think part of the reason we're having problems in the United States is there really wasn't any kind of real public debate because we didn't have any regulation to talk about it whereas in Europe with the, you know, novel food directive, you do have that.

JIM WHITE: Guy.

GUY CARDINEAU: I have a question for Dr. Hansen. It's in relation to the TERRA PRIMA story about the 180,000 bags of corn chips, I guess it was.

MICHAEL HANSEN: Yeah.

GUY CARDINEAU: I testified at the FDA hearing in Oakland in December, and I heard a similar story. A lady from Wisconsin talked about 65,000 bags of corn chips. And as I sat there and listened, I wondered, is it the same story?

MICHAEL HANSEN: Yeah. Depending on -- the numbers are different, but it was 114,000 was the -- different numbers now but --

MICHAEL HANSEN: $114,000 was the economic cost.

GUY CARDINEAU: All right. Well, here's my question. Organic foods cost more because they're organic. So in this instance either there was seed mixing or there was pollen drift theoretically.

But, here's their plot of corn that's organic, and here's their Bt corn plot where they might have gotten pollen drift, or here's their grain storage with their organic, and here's their Bt corn, and they got mixed together.

But now suppose this is a nonorganic field that's been sprayed in which there is no transgene. Now, it suggests to me that what we have here is either bad cropping practices that allow pollen to drift into their organic field because even if it's not a transgene, if that sprayed material drifts over there, that's no longer organic. But I can't determine that and I'm paying more for it.

Or if this sprayed crop is mixed in a seed bin with their material, it's not transgenic, so I can't detect it, yet I'm paying more for that crop.

So it strikes me that what this really showed us was not the problem with transgenics but the problem with organic practices, that they really don't live up to their reputation, and in fact, they're contaminated all the time. It's just that we can't tell.

Can you comment on that?

MICHAEL HANSEN: Yeah. Actually, with the organic role, Consumers Union has been critical of the organic industry. We have said, for example, that there should be required pesticide residue testing because since organic foods do tend to cost more, there might be an economic propensity to cheat.

And that has actually been found in the past. It was found with one case with carrots. They were selling so many -- I think it was Fresh Fields. One of companies were selling to many organic carrots on the Web on the East Coast, somebody actually did some calculations and figured out that the amount that that entity was selling was larger than the certified organic production of carrots U.S.-wide. So clearly, something was going on.

I think you could potentially say that this shows problems with the organic rules, but it's larger than that now because since there are these markets in Europe and now Japan for guaranteed GE free, that's when you're going to have problems of also conventional farmers who are trying to grow these GE-free grains because we're already starting to see a two-tiered grain market appearing.

And so that's actually one issue that is being struggled with by the National Organic Standards Board, and I think if you talk to folks within the organic industry, there really is some kind of concern over what they can do about this pollen contamination issue.

It wasn't a problem in the past because if you got a conventional crop and you had pollen drift, that didn't make the organic crop function nonsellable.

With this GE stuff, that's sort of true now. If you want to sell into the European market, it doesn't have to be organic. It can be conventional, but they don't want those certain GE corn varieties. And the same thing is happening with Japan as well.

So you start to then -- that's going to be a very tricky question. And I'll just say that I notice that the

industry does deal with this. If you look at cotton, for example, in California, they prevented Sally Fox's Natural Cotton from actually growing it in plants there because she has some colored cottons which she's discovered, and they wouldn't let them be grown in California, and the reason is that the conventional cotton growers didn't want any of those genes appearing in any of their cotton varieties and making them not pure white.

So it's an issue that I think is going to have to be dealt with. There's some talk at Glickman's panel of perhaps there's something that needs to be done on crop insurance. But this whole issue of pollen flow has to be dealt with because the economic impacts -- this is not even the ecological impacts but just the economic ones.

JIM WHITE: Before Louise goes, I want to -- Michael, one of the joys of working in federal bureaucracy is reading proposed rules, and I read the 500-page and now officially out for 90-day comment on the organic rule.

I think I'm going to ask Michael. Is the Consumers Union going to comment on the failure of this new AMS national organic rule to address pesticide testing in organics and address pollen flow before the rule goes final?

MICHAEL HANSEN: I'll say in our first comments with the first round which we think were bad, we did actually raise the question of that there needs to be some form of pesticide testing for residues. We said that in our comments in '97 or '98, and there's folks in the organic community that are upset about that. They sort of understand it.

So yes, we will comment on both those things. And I will say that from what we've seen of the rule and going through the organic rule, it's far better than the original rule that came out. It only looks like -- there's some small minor issues, but all the larger ones and even -- there were 50 or 60 that I think folks identified, and there's a much smaller suite here.

JIM WHITE: You didn't address pollen flow.

MICHAEL HANSEN: Well, that's going to be a thing, yes, that needs to be raised, and the whole pollen flow issue -- because we don't know how to deal with it. We do have some evidence from the USDA that there have been some farmers that have asked about it under the federal crop insurance program, and it's interesting because they've gotten letters back that say, "We can insure you against natural disasters, but we can't insure you against manmade disasters." And they likened it, interestingly, to toxic contamination.

JIM WHITE: Louise.

LOUISE HENDERSON: Louise Henderson from the Center for Veterinary Biologics. There's some real issues about risk analysis that are always raised here, and as a regulator, I'm charged with looking at products that are brought to us for testing, and then you can actually do test some of those kinds of products.

But what I would like to have anybody comment on that would like to is how one can determine whether or not one should look at outregulating the expression, thereby limiting the acreage, versus raising the level of antigen. And we all know that toxicity issues are often dependent on concentration of expressed proteins or whatever the biologically active element is.

Once it's in a final product, I'm not so sure that that's so difficult in the testing arena, but do you have any suggestions for how one might go about looking at how we would balance those two concerns?

JOHN HAMMOND: I think if you have something that's being produced that is toxic to the plant it's being produced in, Carole Cramer's system where you produce it after the plant has been harvested is the obvious answer to that one.

If it's not toxic to the plant you're producing it in, I'm not sure that there's very much to tip the balance either way. That's probably a question of the economics of raising the expression level in the individual plant versus planting greater acreage.

I'm not sure that there's very much scientifically to choose there. I think that's more a matter of the economic impact of one over the other.

JIM WHITE: We're going to have two questions. Michael is going to ask a question, and Vldadi gets the last question of the day.

MICHAEL HANSEN: The one question I'd like to ask, since in reading some of the submissions that I did, I notice that -- and in the discussion, John, that you did, you talked about gene flow, but I was wondering, is there much experimental data? Are people looking at horizontal gene transfer from plants to bacteria?

I know in the things I saw, people just referred to an article in 1993 that says that this doesn't happen, and I'm just wondering since it grew apart in 1998 in an article in the Journal of Applied and Environmental Microbiology, we're

able to demonstrate with a Sinetobactor movement of transgenes from transgenic sugar beets into this soil bacteria.

It was in the lab, but as they said, that -- and I'll quote -- transformation of naturally competent bacteria by transgenic plant DNA, even with plant homogenates was demonstrated for the first time.

And so since others have raised the horizontal gene flow issue and not even critics such as myself but on Glickman's panel Marjorie Hoyt from Florida raised that issue that she was concerned.

So I'm wondering, are people doing experiments in the field to look at microbial ecology and to look at horizontal gene transfer so it's sort of the below ground?

I think the stuff that the CDC talked about on wildlife is done very well, but I'm wondering if anyone is looking at the soil and microflora and fauna?

JOHN HAMMOND: I'm going to have to refer that to Jim because that's out of my realm.

WHITE: This discussion is going on recently in the discussion group from Switzerland, and we can't go through that, Michael. I'll send you that. But the best system that I understand is with Erwinia which can grow on potatoes.

Naturally, it causes disease on potatoes. They did testing on that so the Erwinia has to grow on potatoes. That's their own source, and they've shown no horizontal transfer on that, so I think that's a better system than these other systems, but I'll send you that information.

Vldadi.

VLDADI YUSIBOV: I'll just first comment on Allen's question that we've been using transgenic P12 in plants that replicate transgenics for four years now to produce different biological and as Dr. Koprowski mentioned.

And I agree with John that hypothetically it's possible that the replicate may indeed replicate some of the plant RNAs. Practically, it has to improve them.

On top of that we have done some experiments. We did consider the question, but unfortunately, you have such a huge question that which RNA or which messenger to keep and which one to check in order to predict or at least to prove or disprove the replicate indeed does or does not replicate the plant messages.

But a final one we have done, we have purified the viruses from different constructs over the four-year period produced in this transgenic plants trying to see if any of the plant messages are attached with the plant virus by inoculating even with the wild-type virus which has all the components, and we never have been successful, or we never succeeded to recovering the wild-type so-called plant RNA.

It's encapsulates the viral RNA which really rolls out sort of like even if it does replicate some of the messages at some level, but the concern, it's not encapsulated as a part of RNA, and if it's not transmittable to the next -- to the susceptible hosts.

And second, I had a question just for Mike. I think it's more like insect biology question. There was a question about the resistant bugs, resistant to the Bt.

I think there should be some sort of, like, a diversion. What you really had to look into will get into the Bt which can become resistant to the Bt-producing corn, or are we looking into the same problem when you're spraying the insect with the bunch of chemicals where you have much more chances to getting insects which will be resistant to pesticides, herbicides, and you will be facing the problem of not only having a single bond which could be resistant to a Bt. You may face a problem -- I think some of you may remember this better than I do. I think there was a mosquito problem in the Great Lakes. The mosquito population was sprayed with Bt or something like that, that the mosquitoes became resistant.

So in this case I think it has to be discussed, but at least to my view, it will be much more safer. And then we have to go into the mutation rates, which what kind of frequency the bug will mutate, which is really -- I mean question of, like, it's not going to be very frequent.

So we really have to choose and pick which will be the better. I mean you may have the same problem of obtaining the resistant --Circo-Resistant bugs but in a much wider population of the insects by spraying them with the chemicals rather than just having a selective Bt corn which really produces the Bt only in green part of the plant, not in the part we consume, if you can comment on that.

JIM WHITE: I'm going to interrupt. Okay? We're supposed to be here at 4:30. There's a cash bar that's going to be out here, and Vldadi can buy Michael a drink, and they can discuss Bt. And I want to remind everybody they are invited to the banquet that's at 6:30. That's going to be held on the second floor of this building. And if you

want to talk to Michael about Bt-resistant insects, you can do that then.

Thank you very much.

(Meeting concluded at 5:00 p.m.)

C E R T I F I C A T E

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I, SUEANN GRAHAM, a Certified Shorthand Reporter and Notary Public in and for the State of Iowa, do hereby certify that the foregoing is a true and accurate computer-aided transcription of the meeting as taken stenographically by and before me at the time and place indicated on the title page;

That I am neither a relative nor employee nor attorney nor counsel of any of the parties to this action, and that I am not financially interested in the action.

Dated this 16th day of June, 2000.

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SUEANN GRAHAM, CSR, RPR