

THE NATIONAL ACADEMIES

INSTITUTE OF MEDICINE

COMMITTEE TO
REVIEW ADVERSE EFFECTS OF VACCINES

August 26, 2009

Keck Center of the National Academies
500 5th Street, NW
Washington, DC

Proceedings by:
CASET Associates, Ltd.
Fairfax, Virginia 22030
703-266-8402

NOTE: This is an unedited verbatim transcript of the meeting of the Committee to Review Adverse Effects of Vaccine, held on June 24, 2009, prepared by CASET Associates, Fairfax, VA, and is not an official report of the National Academy of Sciences, Institute of Medicine, National Academy of Engineering, or National Research Council (collectively "The National Academies"). Opinions and statements included in the transcript are solely those of the individual persons or participants at the meeting, and are not necessarily adopted or endorsed or verified as accurate by The National Academies.

UNEDITED VERBATIM TRANSCRIPT

Table of Contents

Welcome and Introduction of the Committee	1
The Immune Response to Vaccines and Natural Infection - Neal Halsey	8
Discussion	44
Antibodies, Vaccines, Neuroinflammation, and the Blood- Brain Barrier - William Banks	53
Discussion	85
Metabolic and Other Genetic Syndromes - Bruce Cohen	100
Discussion	135

P R O C E E D I N G S

**Agenda Item: Welcome and Introduction of the
Committee**

DR. CLAYTON: Good morning. As Chair of this study and of this committee meeting, I would like to welcome everyone, including those of you who are listening in by telephone.

The task being undertaken by this committee of the Institute of Medicine is to review the epidemiological, clinical, and biological evidence regarding adverse health events associated with specific vaccines covered by the Vaccine Injury Compensation Program. The vaccines currently of interest to us are varicella zoster vaccine, influenza vaccines, except for H1N1, hepatitis B vaccine, and human papillomavirus. We understand that very soon we will officially be asked to include four more vaccines: hepatitis A, MMR, the meningococcal vaccines, and the tetanus toxoid-containing vaccines, such as DTaP, Tdap, and Td.

In anticipation of that increase in workload, the committee membership has expanded since our last meeting.

I want to note that this is an open public session, and this is the third meeting of this committee. There are more in front of us.

UNEDITED VERBATIM TRANSCRIPT

One of the first goals for this committee is to explore ways to evaluate the biological phenomena that link the injection of vaccine to adverse physiologic and medical outcomes. The three talks we have today will move the committee quite a bit forward in its thinking.

The first talk will be by Dr. Neal Halsey. Dr. Halsey has decades of experience conducting clinical trials of a variety of vaccines, as well as research to understand specific safety concerns for some vaccines.

The second talk will be by Dr. William Banks, who will talk about the blood-brain barrier, specifically antibodies, neuroinflammation, and vaccines and their interactions with normal and aberrant functioning of the blood-brain barrier.

Our final speaker is Dr. Bruce Cohen, a pediatric neurologist with clinical expertise in caring for and understanding the development of children with metabolic disorders. He will most certainly help us think about the potential effects of vaccines in these very vulnerable children.

Each speaker will have 40 to 45 minutes for the presentation, followed by 15 minutes of discussion with the committee.

I want to remind everyone that this is an

UNEDITED VERBATIM TRANSCRIPT

information-gathering session. That is, the committee is in the process of assembling materials that it will examine and discuss in the course of making its conclusions. Therefore, I ask everyone here today to be extremely mindful of the fact that the committee has made no conclusions and that it would be a mistake for anyone to leave here today or to get off the line here today thinking otherwise. Comments made by individuals, including members of the committee, should not be interpreted as positions of the committee or of the IOM. In addition, committee members typically ask probing questions in these information-gathering sessions, which may not be indicative of their personal views.

The committee will deliberate thoroughly before writing its draft report. Moreover, once the draft report is written, it must go through a rigorous review by experts who are anonymous to the committee, and the committee then must respond to this review with appropriate revisions that adequately satisfy the Academy's Report Review Committee and the Chair of the National Resource Council before it is considered an Academy report.

At this point, I would like to ask the committee members to introduce themselves and briefly describe their expertise and affiliation. All members of the committee

but Dr. Marc Patterson and Dr. Polly Thomas are with us.

I'll start with Ellen Clayton. I am the Rosalind Franklin Professor of Genetics and Health Policy at Vanderbilt University. I'm a general pediatrician and a law professor and director of the Biomedical Ethics Center there.

DR. DEL JUNCO: Good morning. I'm Deborah Del Junco. I am an associate professor of epidemiology and clinical and translational sciences at the University of Texas Health Science Center in Houston.

DR. NGUYEN: Good morning. I'm Ruby Nguyen, from the University of Minnesota. I'm on the faculty in the Division of Epidemiology and Community Health. My area of expertise is reproductive and perinatal epidemiology.

DR. BARRETT: Good morning. My name is Doug Barrett. I'm a professor of pediatrics, immunology, and pathology at the University of Florida in Gainesville.

DR. ABAN: Good morning. My name is Inmaculada Aban. I'm associate professor in biostatistics at the University of Alabama at Birmingham.

DR. BIBBINS-DOMINGO: I'm Kirsten Bibbins-Domingo. I'm a general internist and epidemiologist at the University of California, San Francisco.

DR. JOHNSTON: I'm Clay Johnston. I'm a vascular

neurologist and epidemiologist at the University of California, San Francisco.

DR. KOMAROFF: I'm Dr. Anthony Komaroff. I'm a professor of medicine at Harvard Medical School, practice general internal medicine, do clinical investigation, and teach clinical epidemiology.

DR. COLDITZ: Graham Colditz, professor at Washington University School of Medicine and deputy director of the Institute for Public Health at Washington University in St. Louis -- epidemiologist.

DR. WEINER: I'm Leslie Weiner, from the University of Southern California. I'm professor of neurology, microbiology, and immunology. My interest is immune and viral effects on the nervous system.

DR. LAWRENCE: I'm Paige Lawrence. I'm an associate professor at the University of Rochester Medical Center in environmental medicine, immunology, and microbiology.

DR. DIAMOND: I'm Betty Diamond. I'm a researcher at the Feinstein Institute for Medical Research and faculty at Albert Einstein Medical School and rheumatologist and immunologist.

DR. CONSTANTINE-PATON: My name is Martha Constantine-Paton. I'm a professor of brain and cognitive

science and biology at the Massachusetts Institute of Technology and an investigator in the McGovern Institute for Brain Research. My expertise is development of the nervous system, particularly synaptic development.

DR. BEBIN: I'm Martina Bebin. I'm from the University of Alabama at Birmingham. I'm an associate professor in neurology and pediatrics. I'm a pediatric neurologist, with an expertise in epilepsy.

DR. MARKERT: Good morning. I'm Louise Markert, associate professor of pediatrics and immunology at Duke University Medical Center. My area of expertise is primary immunodeficiency.

DR. SAMPSON: Good morning. I'm Hugh Sampson. I'm professor of pediatrics and immunology at the Mount Sinai School of Medicine. My area of expertise is in mechanisms of food allergic reactions.

DR. HALSEY: I'm Neal Halsey, a professor of international health and pediatrics at Johns Hopkins University.

DR. COHEN: I'm Bruce Cohen, Cleveland Clinic Foundation. I'm a pediatric neurologist.

DR. BANKS: Bill Banks, professor at the St. Louis University and VA Medical Center, talking about blood-brain barrier later.

UNEDITED VERBATIM TRANSCRIPT

DR. CLAYTON: Can we have the other people in the room introduce themselves as well?

(Introductions around room)

DR. CLAYTON: Thank you.

Before we begin, I should mention that we have a toll-free phone line open so that interested parties who could to be here in person can listen to the presentations. We have or shortly will have the PowerPoint presentations on the project Web site to help make following the presentations easier for those on the line. They are accessible at www.iom.edu/vaccineadverseeffects. The bottom of that page has a link that will take you to meeting number 3. Click that, and the top right of the meeting page has a link to the meeting agenda. The PowerPoint presentations will be posted at the bottom of that page as soon as we can.

We will not be able to entertain questions from the phone audience, but if anyone on the line has comments about the workshop for the committee's considerations, feel free to email them to Vaccine Safety @ nas.edu. Please note that emails to the committee must be posted in the public access file.

On another note, I would like to ask all speakers to disclose any conflicts of interest with the subject at

UNEDITED VERBATIM TRANSCRIPT

hand. In particular, we would like you to disclose financial relationships you have with those pharmaceutical companies that make vaccines, even if your relationship is not regarding vaccines. We would also like to know if you have financial relationships with HRSA, CDC, or FDA.

We will begin our day with Dr. Neal Halsey. He is professor in the Department of International Health, as you have already heard, Disease Prevention & Control, and director of the Institute for Vaccine Safety at Johns Hopkins Bloomberg School of Public Health.

Agenda Item: The Immune Response to Vaccines and Natural Infection

DR. HALSEY: I'll begin with the disclosures. I serve on data and safety monitoring boards for Novartis, for vaccine studies, and I have in the past served on one for Merck. I believe we are finished with that. I do have a grant, for which I receive no salary, for a doctoral student from Merck for HPV-related studies in Peru. I am the PI on studies in Guatemala, funded by Berna and Intercell, neither of which, I think, has vaccines licensed in the United States.

I do serve as a reviewer for the Vaccine Injury Compensation Program from HRSA. I have grants from CDC for a clinical immunization safety assessment site, one of six.

I also have a grant for studying hypersensitivity reactions to gelatin-containing vaccines, from CDC.

I was asked to review some of the aspects of adverse events associated with vaccines and the human immune response. I was asked nine questions. I would start by saying that almost each of these nine could easily constitute a full hour.

I now understand why you didn't ask a true immunologist to give the talk, because they would easily spend an hour on this first question: Are there noteworthy differences in the immune response to the various types of vaccines?

Yes, the answer is that there are many. There are general rules, but there are many exceptions to these rules.

The answers to this and all the other questions I have are found in the reference book, the textbook entitled *Vaccines*, Plotkin being the senior editor of that book.

The second question -- I'm going to take these two questions together to address -- is, what are the noteworthy differences in the immune response to a vaccine compared to the immune response to the natural infection? Are there generalities or is it vaccine-specific? If it's vaccine-specific, for which vaccines is the immune response

most notably different than the response to natural infection?

Differences between the immune response to vaccines and natural infection:

I'll start with live attenuated vaccines. Qualitatively, in general, they are similar to the wild-type infection. They are both infections. Quantitatively, there is often a decrease in the antibody response following a vaccine. Both induce cell-mediated immunity, as well as humeral responses, as measured by serum IgM, usually. We need to remember that with natural infections and also with live vaccines, the host is exposed to numerous antigens, but we are often only measuring the response to one, or perhaps two at the very most, for vaccines.

A bacterial infection stimulates responses to many different antigens. I believe there are more than 50 that have been measured in response to group A strep. Many other bacteria, such as pertussis, induce probably equally as many. We have not looked for all of those responses.

In vaccinology, we are interested in identifying correlates of protection. That's why we measure only, usually, responses to single antigens. Serum IgG is the usual correlate of protection.

This is not a list of all vaccines. This is a list taken from the chapter in the Plotkin textbook on "Vaccine Immunology" by Claire-Anne Siegrist, which I highly recommend to anybody who wants to get into depth in this.

Live viral vaccines also induce T cell-associated immunity, usually mediated through CD8 cells. This has been measured with regard to some vaccines.

The antibody tests that we do vary by the vaccine. Neutralizing antibody is looked at for measles, respiratory syncytial virus. Most of the time, though, we are measuring antibody to a surface antigen or a toxin. We look at hemagglutinin for measles and influenza, which is expressed on the surface of those viruses. For polysaccharide vaccines, we look at anticapsular antibody. *Haemophilus influenzae*, pneumococcus, and meningococcus are three.

Other times, we are looking for a specific protein, an individual protein on the surface, such as with HPV vaccine. For others, we are looking only at antitoxin antibody. We have purified toxoids, tetanus and diphtheria. Then, with pertussis, it's one of the key elements in the new acellular pertussis vaccines.

I need to remind you that the type of antigen is

only one of multiple vaccine-associated factors that influence the response. The dose, the number of doses, the interval between doses, the route of administration, and adjuvants all affect the host immune response. Plus there are at least eight different factors in the host that influence the immune response -- age, gender, smoking, genetics, body mass index, prior exposure to either the vaccine antigen or any component of the vaccine. Passive antibody acquired from a mother or administered therapeutically can influence the response; and underlying immune deficiencies, whether they are primary or secondary, from therapy such as cancer, and some drugs have selective effects that blunt the response to vaccines, such as chloroquine.

I was always fascinated by an observation we made in Haiti in 1982, when no measles vaccine had been introduced in that country. In studying the mothers of children, we note at least a 32-fold difference in the height of the antibody that these mothers had from natural infections, with some of them having very low antibody responses and some fairly high responses.

The first measles vaccines were licensed in 1963, after nine years of passaging through more than 60 different passages in human and animal cell lines to

produce the vaccine, which did attenuate the vaccine. The first vaccine we had was the Edmonston B. I'll point over here at the left screen, where you can note that there was a marked reduction in the incidence of moderate to high fever and rash through that attenuation. Administering it with immune serum globulin -- or gamma globulin, as it was referred to -- reduces the rates of those adverse events. Subsequently, five years later, further attenuated vaccines were made through further serial passages that did not require the administration of immune globulin. But you still see low rates of fever and rash associated with the further attenuated vaccine.

Krugman compared the immune response to natural infection with measles over time, as shown in the green line on this figure, and then compared it to the response to Edmonston B in children that he had in longitudinal cohort studies. You will note that basically these figures overlap, so that even though there was attenuation of the virus, they still induced basically the same level of immune response to measles hemagglutinin. With further attenuation, as shown in the lower line, the blue line, there was a decrease in the antibody response -- approximately a fourfold lower geometric mean titer with the further attenuated vaccine as compared to the natural

or wild-type virus infection.

Krugman also noted the same log-normal distribution of antibody following attenuated vaccine. That stayed the same through time, but in this situation, some, initially, usually low responders got to very low levels -- in fact, undetectable antibody after 14 years.

Comparing measles, mumps, and rubella vaccine, and the antibody response with just measles vaccine alone, you see a similar response to the measles component. This is not the MMR that's being used in this country. It's being used in another country. But note also, with measles vaccine compared to natural infection, a small percentage, 2 to 5 percent in different studies, of children don't respond, even when they are past the time when maternal antibody is not present. The same is true for mumps and for rubella vaccine in the combination product.

In making these vaccines, they had to adjust the titer of the rubella and the mumps in order to get adequate responses compared to what they had with the standalone vaccines. Measles they didn't have to adjust.

Live viral vaccines quantitatively often induce a lower response than you see with the natural infection, but you do get very high levels of protection with a single dose of vaccine. A second dose is recommended for most

live viral vaccines, primarily to cover the initial non-responders, but waning immunity does occur with several live vaccines.

The mumps vaccine is less effective than the other live viral vaccines. We now know from long-term follow-up studies done especially with our outbreak in 2006 that the efficacy is less than we had earlier thought. Even with two doses, there is some evidence of waning immunity for some people, but not most, after mumps vaccine.

We also have learned in the past three years that there is some evidence for waning immunity following varicella vaccine, after a single dose of varicella vaccine, with increased breakthrough rates, as shown in the solid bars on this curve, with time after vaccination. A second study demonstrated that five or more years after vaccination, the rates of vaccine failure were significantly higher than the rates in the first five years -- further evidence. And there is a third study that now shows that there is some waning immunity following varicella vaccine. We are now giving a second dose of varicella vaccine.

Other types of attenuated vaccines are made. The rotavirus vaccine and the live attenuated influenza vaccine

are made through reassortant technology, where they have segmented genomes. In this case, there are 11 chromosomes. I made an error in the slides that are on the Web site. I apologize for that. With the reassortant technology, they are able to make vaccines that have nine of the original animal -- in this case, bovine -- rotavirus, which carries with it the attenuation characteristics, so that you have minimal signs of infection. Yet they can introduce the human genes for surface proteins that protect against human infections.

We have exceptions to the general rules I gave you with some live attenuated vaccines. This is a *Salmonella* Ty 21a bacterial vaccine that is replication-deficient -- a newer technology that is being used to make vaccines. This bacterium does not contain a key enzyme, UDP-gal-4-epimerase. I'm not going to go into how it works. Also there is 80 percent reduction in two other enzymes. This vaccine does not produce the capsular polysaccharide antigen, which is important, actually, in pathogenesis, but protection is mediated through other immune responses from replication in the intestine. It takes three to four doses of a fairly large number of bacteria to induce moderate to high levels of protection from this vaccine. So this is qualitatively and

quantitatively different from wild-type infection with the original parent organisms.

Killed or subunit vaccines don't replicate. Therefore, there is usually no CD8 stimulation and long-term immunity. We often have bacterial vaccines that have just limited antigens. There is a *Salmonella typhi* vaccine that contains only the Vi polysaccharide, and it does protect. I mentioned earlier toxoids. You don't get all of the other proteins, for the most part, in these toxoid vaccines. You only immunize against the toxin, which is responsible for most of the disease. I have already talked about surface proteins and acellular pertussis as examples.

General rules for killed vaccines:

- It takes multiple doses to induce immunity.
- We induce IgG antibody, which is primarily what is associated with significant protection.
- There is waning immunity for almost all killed vaccines, and booster doses are generally needed -- again, the general rule.

A classic diagram by Arturo Galazka depicting the response to tetanus toxoid. It takes at least two doses to get above the protective level, and there is waning immunity. With multiple doses, you get longer-lasting protection. He has exaggerated it a little bit in this

diagram. It's not quite as bad as is shown here, especially after three and four doses that are there. But it depicts the pattern.

There are exceptions. This is hepatitis A vaccine. For reasons that I don't understand, a single dose induces immunity in 90 to 100 percent of children; a smaller percentage in adults, but still close to 90 percent. We only give two doses, separated by six to 12 months. You get very high levels of antibody that last for a long period of time.

If we look at this figure on the left-hand side, the immune globulin that we give for passive prophylaxis against hepatitis A, which does protect, induces low levels of antibody. This is a diagram from Stanley Lemon, who can think in terms of natural logs. I can't. This is on a metrolog(?) scale, but still it's log-based. There is an attenuated vaccine, not used in this country but in Asia, that induces higher levels of antibody. The inactivated vaccines, the two of them that we have, both induce higher levels of antibody, but these are still a couple of logs lower than the wild-type infection. This depicts the pattern that helps us understand these vaccines.

With some single-protein vaccines, such as the L1 protein in HPV vaccine, they are manufactured in such a

way -- and this is unique -- that they can take just one of the surface proteins on the virus and then, when they make them, they self-assemble into a viral-like particle that is very important in inducing high levels of antibody and protective levels of antibody. Now studies are showing that these induce even higher levels of antibody than the natural infection does and they persist very well for at least five years. Long-term studies are now in place to determine how long they will persist and if and when we will need to give booster doses.

The third question that was asked: What are the qualitative and quantitative differences in immune responses to vaccines for specific age groups -- preemies, neonates, infants, children, adolescents, adults, and elders?

I'm going to have to be abbreviated in terms of addressing this, because there are many differences.

Infants we know a great deal about now. There are immature responses to multiple antigens. If you immunize early, you often get shorter durations of protection than you do with immunizing later. We have oftentimes decreased affinity maturation, so the antibody is less effective. We often have interference by passive maternal antibodies.

This is an old slide that I copied about 25 years ago. I couldn't find the original reference for this, but it's from Heikki Peltola in Finland, where they studied the plain *Haemophilus influenzae* polysaccharide vaccine. We don't use this anymore, but it was the first licensed vaccine. This shows the response by age in months, with an increasing percent response with time. By 18 months, in Finland, the majority of children developed what was considered to be adequate antibody to provide protection for at least a period of time.

But polysaccharide vaccines are notorious for inducing poor response, especially under 18 to 24 months of age. They do not go through the same process that we see with protein-based vaccines. They are called T-independent responses. They can stimulate B cells to respond and differentiate, to produce either IgG, IgM, or sometimes IgA. They don't get into the germinal center, where they go through all of the T cell-associated responses. The immunologists on this committee can talk in more detail about this than I can.

Also when we give multiple doses, there is rapid decline in antibody, especially in the first year of life, so that the antibody levels decrease back to baseline -- what the general population that has not been immunized

has -- repeatedly. There are not long-term memory cells stimulated. So the polysaccharide antigens are not very effective under two years of age.

With measles vaccine, we normally don't give that vaccine, in the United States, until 12 to 15 months of age, but in developing countries, at nine months, and then under circumstances of increased exposure, as early as six months. But there is interference by maternal antibody which blocks the replication of this live virus, as the primary method. There are also some other factors associated with immaturity. So we get poor responses by age. These vary in different populations, largely based upon the difference in the rate of loss of maternal antibody. That varies by country, as depicted by Francis Black in 1980, in a comparison of several different studies. My own studies and others have confirmed this difference that is there.

We give different vaccines at different ages. We can give at birth BCG vaccine, oral polio vaccine, hepatitis B. We wait until six weeks of age to give most of the routine vaccines that we give to children. If we give some of these too early -- the *Haemophilus* or the DTaP -- we actually blunt the final response to the vaccine, even if we give all the other subsequent doses.

You can interfere to a certain extent with the take rate by giving too early to some antigens.

I mentioned already that nine to 12 months is the earliest age for measles and yellow fever. With yellow fever, even when there is no maternal antibody, we don't want to give this earlier than nine months because in this situation there is an increased rate of a severe complication, encephalitis, associated with this vaccine. We won't go into that in more detail; we'll mention it later.

Twelve months of age is the earliest age for hepatitis A vaccine, because even small amounts of maternal antibody blunt the response to that vaccine.

Again, we wait until 24 months for plain polysaccharide vaccines.

We can give oral polio vaccine, which is a live replicating agent, at birth because it replicates in the gastrointestinal tract, where there is less interference from passive maternal antibodies that most children receive.

Again, the immunologists would go into more detail. Claire-Anne Siegrist lists a half a dozen different factors associated with immaturity in infants. I would direct you to that chapter to read in more detail

about this.

We also see differences in the response to natural infection by age, as depicted by hepatitis B, where if you are infected at birth, you have a very high rate of becoming chronically infected. You never get rid of the virus. The likelihood of becoming chronically infected decreases with increasing age, until it's about 8 to 10 percent in older children and adults.

The opposite is true with regard to the symptomatology associated with infection. Most infants infected at birth and the first few years of life are asymptomatic. You never know they are infected. But with increasing age, they develop increasing likelihood of having clinical hepatitis. We believe this is the host response, the inflammatory response, to the infectious agent that causes much of the hepatic damage.

After age 50, studies have shown, with several different vaccines -- some of the better studies with hepatitis B, influenza, and pneumococcal polysaccharide -- that there is a decline in antibody response. We all lose cells, CD4 cells in particular, with increasing age. But, in fact, there are differences and declines in the geometric mean antibody response by age, so that children 9 to 15 years of age actually get higher

levels of antibody than do 16- to 26-year-olds. These are all females. The ones in the pink area participated in the efficacy trial for the licensure of the Merck HPV vaccine. On the left side are studies that were done, called bridging studies, to demonstrate that, vaccinating at this age, you can still induce very high levels of antibody.

I like to say that by the time we get our driver's licenses, we are already past our peak antibody response capability, to vaccines at least.

Claire-Anne Siegrist talks about aged individuals in the chapter. I suspect that I am now aged. I'm not sure what cutoff point, and she did not give a cutoff point when she talks about it here. There are other factors, other than the natural loss of CD4 cells and others, that contribute to this decline in the immune response with increasing age. Again, you have immunologists here who can go into this in more detail than I can.

Fourth question: Are there special populations, other than age or the severe immune deficiencies, who respond qualitatively differently to vaccines and/or natural infections?

I would encourage you to look hard at females. This is just one example. Females have higher rates of adverse events associated with rubella vaccine, especially

post-pubertal females, where you can have 30 to 40 percent or more with joint symptoms, both arthralgia and arthritis. These are four to five times higher than are noted in males. There are differences also in the reported joint and other symptoms associated with Lyme vaccine, in those studies with influenza vaccine, and now with anthrax vaccine, most recently studied. There are some differences in the cytokine responses to vaccines. It's not just reporting artifact that is occurring here.

With pregnancy, there are increased rates of complications from a number of different natural infections. Best studied are both hepatitis A and B, with fulminant hepatitis, influenza, and smallpox -- increased complications. There are immune factors associated with pregnancy that contribute to the increased likelihood of severe disease. Patients with mild forms of immune disorders also have differences in the response rate. HIV-infected -- less likely to respond and shorter duration of protection when they do respond. Patients with underlying complement deficiency or patients without spleens are at increased risk, particularly of meningococcal infections, but also pneumococcal infections, especially for asplenic individuals.

We see increased severity of some diseases in

UNEDITED VERBATIM TRANSCRIPT

developing countries. I know the most about measles. A recent paper by Stan Schulman in *Peds Infectious Disease Journal* highlighted the 10 to 30 percent case-fatality rate that happened with measles in Hawaii in 1848, when it was first introduced, and in Fiji, a 40 percent mortality rate. But we know that children with vitamin A deficiency in developing countries have increased complication rates, including mortality. Crowding is also associated with increased mortality. We believe HIV infection is as well.

This is a summary of studies done in different countries in the 1970s and early 1980s, showing the high case-fatality rates in developing countries -- 30, 35 percent in some countries, 3 to 10 percent in others. For the U.S. -- you can barely see the line there -- it's .3 percent or 3 per 1,000. So there's at least a 100-fold difference in the case-fatality rate from natural infection in different populations. The global estimate in 2000 was that there was about a 3 percent case-fatality rate. Studies with Chris Sudfeld, a doctoral student working with me, have documented that there still is at least a 100-fold difference in case-fatality rate for community-based studies, as shown by the blue dots here, in more recent years.

Vitamin A deficiency is associated not just with

increased mortality, but increased rates of blindness due to severe corneal scarring associated with the infection and secondary infections in the cornea.

Children who acquire measles infection from a household contact have higher case-fatality rates than children who acquire it from a community contact, as depicted in a number of studies put together by Peter Aaby here, with two- to fourfold increased case-fatality rates. This is undoubtedly the inoculum effect. This has been shown with other infectious diseases. The higher inoculum you get, the higher the rates of complication. Situations where there is interpersonal crowding contribute to increased complication rates.

We have also seen what we think is a similar phenomenon with high-titer measles vaccine. Twenty years ago, I and others did studies with high-titer vaccines -- a 100-fold increase in the amount of virus in the vaccines -- in several different developing countries. These are the studies from Senegal, showing that girls who receive the high-titer vaccine had decreased survival, as shown in the bottom line, the yellow line here, as compared to girls who had the standard-titer vaccine. But there was no difference for boys. We still don't have an adequate explanation for this, but this pattern was observed in

three different countries with high infant mortality rates. There was no increase in mortality associated with this vaccine in girls or boys in at least four other studies, including studies in the U.S. We believe that there was interaction between the high-titer vaccine and the immune system that altered the response to other multitude of infections, on top of, possibly, malnutrition that's occurring in these populations.

I'm not going to show the data. We did studies on lymphocyte function. Even 18 months after vaccination, there were some population differences in the delayed-type hypersensitivity responses to a number of different antigens in girls who got high-titer vaccine.

The good news is that standard-titer measles vaccine in developing countries is not associated with any increased rates of complication in malnourished or vitamin A-deficient children.

Children with eczema have increased complication rates from smallpox vaccine. These children have disorders of their immune system, such that they have high concentrations of IgE and they have altered T-cell activity, especially in the skin. They suffer from this complication. Also there is some increased mortality in that population.

These children are not at increased risk for complications from other vaccines, other than the potential for allergic reactions to vaccine components.

Other populations with different responses to vaccine:

- I mentioned HIV-infected.
- There are other mild immune deficiency disorders that are under investigation by our CISA network.
- People who have had their thymus irradiated or removed are at increased risk of severe complication from yellow fever vaccine. They get a viscerotropic syndrome that basically mimics wild-type yellow fever, with about a 60 percent case-fatality rate.
- Cigarette smokers have decreased antibody responses to multiple different vaccines. Exactly how that works I don't know.
- You are going to hear about underlying metabolic and mitochondrial disorders from Dr. Cohen in a moment, so I won't say any more about that.
- Interestingly, in the vaccine field, Finland is a good place to do studies, because they all respond very well and they get high levels of protective efficacy, higher than we see often in other countries, in the U.S. and developing countries in particular.

- Also patients who have hypersensitivity to a vaccine component do respond differently.

Hugh Sampson is here. He can talk in more detail about this. We see immediate hypersensitivity reactions that are IgE-mediated, due largely to various allergens that are present in some vaccines. This is a qualitatively different kind of immune response than you would see with natural infections.

I don't think I'll go through the pathogenesis of the immediate hypersensitivity.

We see delayed hypersensitivity reactions. This is after smallpox vaccine, erythema multiforme. We see this after other childhood vaccines. I have seen it after varicella vaccine and occasionally after inactivated vaccines. The exact pathogenesis of this and which antigens are responsible I don't know.

The fifth question: What is known about genetic susceptibility to differences in the immune response, particularly the qualitative differences?

I would say that qualitatively we know very little -- I know very little -- but that much more has been learned about the quantitative response and genetic differences. This is a field that is rapidly evolving. There are a number of different studies under way. A lot

of focus has been on MHC class I and class II molecules because these are the signaling molecules in the chain of immune processing that goes on in response to either natural infection or vaccines.

Going back 20 years ago, one of the first key studies was done looking at the response to hepatitis B vaccine, and certain HLA types were associated with no response or a very poor response, as compared to people who responded much better.

In other studies more recently -- this is hepatitis A vaccine -- after controlling for female gender, which is associated with an increased antibody response and smoking, which is associated with a decreased response, in regression analyses, one particular HLA allele stood out. There are others before they adjusted for all of these other factors.

Other studies have been done. This is measles, Greg Poland's group at the Mayo Clinic finding particular HLA class I and class II alleles that are associated with either decreased or increased antibody response.

So we are gaining some understanding. We are not down to individual gene level, but we are gaining an understanding of the genetics of the immune system. But it's not simple. It's going to be relatively complicated,

and there are multiple that affect the response.

We do see differences in the immune response or efficacy in different populations. There is a minor correction from the slides that are on the Web site here, in terms of the percentages. But most notably, one of the very first conjugate *Haemophilus influenzae* polysaccharide vaccines, PRP-D, which we don't use anymore, had a 90 percent efficacy in Finland, but Alaska natives only had a 35 percent efficacy.

Rotavirus vaccines: Also we saw higher efficacy in some populations, Finland more than the U.S., the U.S. more than developing countries. There are factors that tend to interfere.

There are a number of other genetic studies that are under way. This is a review in 2001 identifying differences in HLA alleles that are present in different populations and different ethnic groups within the U.S. Also there is a nice summary here of some data from different countries. The authors here speculated that someday we may be making specific and different vaccines for different populations. That hasn't been borne out. I don't believe that that's likely to be the case. It's not very practical to do. For the most part, the vaccines that we have induce high levels of protection. We deliberately

give vaccines in a manner that will capture the vast majority of the population and not just the good responders. You also do what you can to make sure the vast majority respond to the vaccine.

The sixth question: What is the current thinking about how specifics of immune response could predict adverse-event occurrence?

We know some about some adverse events, such as immediate hypersensitivity and IgE. We have a laboratory test there that can be used to investigate and determine what the antigen was that they were responding to and determine that this is, in fact, exactly what happened.

Delayed-type hypersensitivity studies are under way. But we don't have any specific markers that I'm aware of that would help us understand many of these delayed-type hypersensitivity-associated adverse events that are there. That's what we would all like to have in the field of vaccines and vaccine safety, specific markers, so we can determine causality and we can determine whether or not, in fact, this was a coincidental event versus a true cause.

The one event that people are all thinking about now was the 1976 swine influenza campaign that was associated with an increased rate of Guillain-Barre syndrome in the eight weeks following vaccination, the peak

in the second and third weeks. Of course, the questions are being asked now about other influenza vaccines.

In summary, ever since then, if there is an increased risk, it's in the rate of about 1, maybe 1 to 2, per million, inconsistent with different years -- at least a tenfold lower rate than was seen in 1976, when it was about 1 in 110,000.

The pathogenesis of Guillain-Barre syndrome -- they are divided into two groups, demyelinating and axonal. The axonal form is associated with antibodies that interfere with the transmission of active potentials along the nerve. You can actually elute off those antibodies with high doses of intravenous immune globulin, which is used for therapy, and you can get clinical improvement. So at least we have a better understanding of the potential pathogenesis. Specific studies were not done in 1976. We don't have good evidence of knowing exactly what was responsible, whether this is cross-reacting antibody or what, in that case.

There are active investigations looking at a number of neurologic events that have occurred and others that have not necessarily been shown to be causally related to vaccines -- acute disseminated encephalomyelitis (ADEM), transverse myelitis, amyotrophic lateral sclerosis, and

brachial neuritis.

The Vaccine Safety Committee of the IOM did conclude in 1994 that the evidence favored acceptance of a causal relationship between brachial neuritis and tetanus toxoid. But we don't really know what the pathogenesis is there. I'm puzzled by it. I'm curious. It would be nice to have a way to investigate those. Again, we just don't have specific lab markers that can be used to investigate these.

I would point out a couple of other interesting phenomena that have occurred in the history of vaccines.

In 1963, both live and inactivated vaccines were licensed. The inactivated vaccine was given in three doses. It produced hemagglutinin inhibition antibody responses that were protective, and there was protection for up to two years.

However, waning immunity occurred. This is a child 12 years after vaccination, who, upon exposure to measles, developed atypical measles, with different distribution of the rash. You see petechiae with it.

This is not this child, but another child with pneumonia, and an atypical pattern of pneumonia, more nodular pneumonia.

Ten years ago, Fernando Polack and Diane Griffin

at our institution, I think, determined the pathogenesis of this event. They showed that not only did the antibody and immunity wane, but the antibody that was produced by these original vaccines was of low avidity. It was not as efficient or effective as antibody from natural infection or live attenuated vaccine. There was no cytotoxic T-cell response. Upon re-exposure, these children still had some long-term immunity, but it was not protective. Antibody was produced and there were immune complexes and eosinophils deposited in the lungs of animals. We believe that this is the pathogenesis of this event, which occurred many years after vaccination.

Also in the 1960s, formalin-inactivated respiratory syncytial virus vaccines were developed that were associated with increased severity of disease nine months later, after the children were exposed. Rates of pneumonia -- 69 percent in the vaccinated versus 9 percent in unvaccinated. A second study showed 80 percent hospitalization for the vaccinated versus 5 percent who had received a control vaccine.

So an enhanced disease associated with prior vaccination can occur. Obviously, neither of these vaccines is used in any country today.

The seventh question: Are corticosteroid levels

tested on any children after vaccines?

I'm not going to show the data, but they are definitely increased after BCG vaccine. The reference is here.

There have been only a couple of other studies that I'm aware of. This is a study by behavioral scientists looking at salivary cortisol after childhood inoculations, one to four inoculations at well-child visits. We can presume that these are DTaB, Hib, IPV, and hepatitis B. The behavioral scientists didn't think it was important. They considered the injection, probably, to be the stressor event in this case. But there are increases in salivary cortisol within minutes after vaccination and then they start to decline by 30 minutes.

A second study in children and their mothers also showed increased salivary cortisol. This is 27 minutes after vaccination with DTP and Hib. I think it's probably DTaP, not the whole-cell DTP, but I can't remember. They did say DTP in the paper.

The mothers did not have any significant difference.

There was some effect by maternal comforting on the behavioral, but not on the cortisol levels.

In adults, the plain polysaccharide *Salmonella*

typhi Vi vaccine, which actually induces very minimal side effects for the most part, was also studied. The authors -- also behavioral scientists -- reported that the cortisol level was significantly increased, but they didn't put the data in the paper. They were more interested in the physiologic effects. They showed increased rates of systolic blood pressure, diastolic blood pressure, and heart rate occurring. They also looked to see whether there was an additive effect with psychological stress, which is depicted in the top lines here. The psychological stress was telling these volunteers for the study, on very short notice, that they were going to have to give a speech in front of a critical audience, which they did, and it did increase their stress level, it appears.

They also noted an increase in IL-6, an inflammatory cytokine. There are other studies showing that we do see inflammatory cytokines within minutes to hours after vaccination, with several vaccines. This is a topic of interest for study for vaccine safety. We think these inflammatory cytokines are probably associated with some of the fever, myalgia, and perhaps even the arthralgias that are occurring.

The eighth question: What parameters are examined in clinical trials of vaccines?

This was difficult because there is so much variability. I think probably everybody here knows that safety and immunogenicity are looked at in all the pre-licensure, Phase I, II, and III studies. Phase III studies also evaluate efficacy. There is usually a subgroup for immunogenicity so we can get these correlates for protection. Phase IV post-licensure is designed usually to look for either safety or effectiveness, and occasionally for both. FDA is basically requiring this now. For almost all recently licensed vaccines, you will see Phase IV studies.

The immunogenicity parameters that are measured in these studies are usually based upon human or animal correlates of protection against wild-type disease, and the specific assays that are done, usually antibody. We have already talked about those. Sometimes CMI studies are being done as well.

Safety studies vary by the phase. Phase I studies: in-depth, daily visits, telephone calls, diary cards, and so forth, asking about everything that happens to these individuals. Sometimes there are additional questions, where people ask because of some hypothesis about the wild-type agent or the vaccine possibly causing them, that come out from either reports of the disease or

initial reports from the vaccine. So there is no standard set. Each study is designed to carefully collect serious adverse events, but also common milder adverse events that may be associated with the vaccine.

By the time we get to Phase IV, we are really just looking for serious adverse events. You don't want to collect information about every little thing that happened to people. Post-licensure, we also do special studies to investigate special questions.

I really can't be a lot more specific about this. We could pull individual studies and go into in-depth detail, but I don't think you need that, unless you get into specific vaccines.

The last question: Could there be a difference in reactions if inoculation was spread over a longer period? Please summarize current information regarding any studies that have made this comparison for a particular vaccine, if I'm aware of them -- MMR, MMRV, polio vaccines, Hib, and DTaP.

I'll show just a few examples.

With the attempts to combine DTaP with Hib, there were studies done by at least four different manufacturers in this country. A couple of those studies did show that there were some increased rates of local reactivity. This

is the local erythema, with the DTaP/Hib combined in the purple, compared to the two different products administered separately, in the gray and the green. You can see that there are slightly larger reactions, but statistically significant, with the combined product, with the first dose; not significant after the second and the third dose in this study. There were no differences in serious adverse events with the combination products.

With the whole-cell DTP with the Hib, there were no increases in the rates of side effects. Those products were licensed and in use until they were replaced by the DTaP.

Immunogenicity studies, however, with the initial products made by several different manufacturers showed lower responses to the Hib, as shown in the right-hand side in the orange, as compared to when the Hib was administered as a separate product. This led, in the United States, to those initial products not being licensed in this country. But they were approved in several European countries and, for the most part, were shown to be effective. Now we do have licensed products. The manufacturers have figured out how to get around this.

There are no increased rates, especially of serious adverse events, with this combination product

versus those administered separately, in any of the studies that I'm aware of.

With measles, mumps, and rubella, the rates of fever, rash, and joint pain, or arthralgia, are similar to the rates that you would see with the monovalent preparations. It is, of course, the measles component that's responsible for most of the fever and the rash and the rubella that's responsible for most of the arthralgia.

I did find one reference to a study done in the late 1960s -- but I didn't pull that paper -- that mentioned that there was a small increase, perhaps, in fever with the MMR compared to them separately. But I haven't reviewed those data, so I don't know.

More recently, with the efforts to combine MMR with varicella vaccine, some interesting observations were made. This is a pre-licensure study, one of the studies that led to licensure. They noted that there was a slightly higher geometric mean antibody titer to measles in the combined product than the separate product, even though the measles component was the same -- the titer was the same, the strain was the same, and the production was the same. Also there was a higher rate of fever with the MMRV compared to the MMR and V administered separately, but at the same time. There also was a 1 percent increase in

rash. I didn't show those data.

In order to combine the varicella with the MMR, they had to increase the titer of the varicella component by tenfold, to about 44,000 plaque-forming units from around 4,000 that were before. That's because in the earlier efforts there were lower immune responses to the varicella when it occurred.

Just in this past year, two studies have been completed, post-licensure studies, with large numbers of individuals. This is more than 300,000 children studied in Northern Kaiser Permanente, in one of the Vaccine Safety Datalink studies done by Nicky Klein and her colleagues. She showed that the rate of fever following MMRV, as shown in the top line, is greater in the seven- to 10-day window of time after vaccination than the rate following MMR and varicella administered separately, as shown in the pink, or the MMR, as shown in the yellow. There was no increased rate of fever associated with varicella vaccine.

This is actually another slide of the pre-licensure data. I'm going to skip that, in the interest of time.

They found a twofold increased rate of febrile seizures in that seven-day window of time. This is a serious adverse event. It did require medical attention.

The attributable risk here is about 1 in 2,000 vaccinated children.

A second study was done in Kaiser Southern California -- this is one of the studies that I serve on the Data and Safety Monitoring Board for -- which also showed about a twofold increased rate of febrile seizures in either the 5 to 12 days, which is what the primary target was there, or the 7 to 10 days that was shown there. The second study did not show an overall increase in the 30-day period following vaccination, but the VSD study did. This is an interesting phenomenon now, where combining them did lead to some increased rate in at least one serious adverse event.

Other combination live viral vaccines: This is trivalent oral polio vaccine. I show it to illustrate the fact that following the first dose of vaccine -- and this is in the United States, where we did get excellent take rates from this vaccine -- you get the best response to type 2. This virus replicates faster, appears to have greater affinity for the cells of the intestinal tract. Multiple studies have shown the same thing. There is a lower response rates to type 1 and type 3 with the first dose, but with subsequent doses you fill in the gaps and you catch up -- close to 100 percent response with three

doses of trivalent oral polio vaccine to all three types.

WHO recently worked to get monovalent OPV licensed, because in developing countries there is a marked decrease in the antibody response and protection associated with the trivalent oral polio vaccine, as depicted in the pinkish-orange color here. This is the proportion of children protected by dose administered. You can see we are at 10 doses out here, and we are still at only 70 to 80 percent. This is Uttar Pradesh, India, where the lower responses were noted.

With the monovalent type 1, you get a better response rate, with even a single dose or with multiple doses.

So it's theoretically possible that there could be an increased rate of vaccine-associated polio -- the question you asked me about -- with individual doses of the monovalent vaccine versus the trivalent vaccine in developing countries. The data are not there, at least from India, to document or support this. This is a hypothesis. I know WHO is looking at it carefully. In the countries where this is being looked at, they have not shown us any data to show that there is an increased rate.

But if it is true, it might be because you get a better response rate. You have less interference than with

the type 2 to the vaccine. Whether there are any other factors that might contribute to this I don't know.

I have already made these points, so I don't need to show that.

In summary, you have asked a series of complex questions. There is a lot of variability in the diseases, in the populations, and in the vaccines. There are some general rules, but there are many exceptions to those general rules.

I don't know how I did on time. I was trying to keep within 45 minutes.

DR. CLAYTON: You did very well, particularly given the wealth of information you just shared with us. Thank you very much.

I would like to open the floor now to members of the committee who have further questions that they would like to ask of Dr. Halsey.

Agenda Item: Discussion

DR. DIAMOND: When you serve on a data and safety monitoring board for vaccine safety, do you have a set of general principles for causality? Most of these come back with "event related to vaccine," "not related to vaccine." Are there general principles? Are there vaccine-specific principles, based on something about the vaccine itself?

What guidelines?

DR. HALSEY: You have asked one of my favorite questions. I actually believe we should have -- and I am actually drafting such general principles for our Clinical Immunization Safety Assessment Network. We are two years into it and fighting over -- I shouldn't say that. Victor, don't take any notes on this.

I think there are. I have published one paper on this, in *Seminars in Pediatric Infectious Diseases* in 2002.

But the best answer to your question is no. They are not out there. They are not commonly used. Especially when you are doing clinical trials, the responsibility of the investigators is to gather the data. The investigators also assign a probability of a likelihood of association. That's part of what they do. The FDA requires them to do that. These are all reviewed by the Data and Safety Monitoring Board. I can tell you that the vast majority of them are possible or unrelated. Many times you can have strong evidence of it being unrelated.

So there are some categories that FDA provides, that WHO provides for how these are classified. But there actually is pretty good science that can be applied.

In data and safety monitoring boards, which are usually done for unlicensed products for which we have very

little data, you don't have enough numbers during the course of the trial usually to determine if there's an increased risk. It's the responsibility of the data and safety monitoring boards to monitor very carefully the comparison groups, and if there is evidence of an increased risk, then you have to weigh the severity of the adverse event, the likely consequences, and so forth. It's the responsibility of the DSMB to stop the trial before it was intended to be stopped.

The best example of this is with the HIV vaccine trials that NIH was conducting in developing countries. The DSMB stopped that trial prematurely because there was evidence of an increased likelihood of infection in certain groups associated with the vaccine.

Similar things have happened many other times. The committee is usually weighing epidemiologic evidence, based upon probability, and statistical analyses. But sometimes studies have been stopped for small numbers of adverse events when they are extremely unlikely to have occurred by chance and you have some biologic plausibility for a causal relationship.

We would like to have general principles for the conduct of a data and safety monitoring board. These are oftentimes written out ahead of time. But they are not

fixed in stone, and they shouldn't be fixed in stone, because you need to empower the committee to go beyond what's there.

DR. DIAMOND: Is there any evidence from any of these studies that adverse events are actually related to antibody titer or immunogenicity? Are those more likely to have an adverse event, those who develop higher titers?

DR. HALSEY: I'll talk two examples. One is the yellow fever vaccine-associated viscerotropic syndrome. Even though they develop widespread infection, my understanding is that, with the small numbers that have been looked at, they didn't develop unusual antibody titers.

You have neurologists and neuroimmunologists here. But when you see encephalitis from natural disease, you find antibody in the cerebrospinal fluid as evidence of production in the central nervous system. With the rare complication from measles, subacute sclerosing panencephalitis, SSPE, which I studied for a period of about seven years, 10 years ago, we did see increased antibody titers. These are children with persistent infections, so they are constantly being exposed to the antigen. So that's one situation where it was.

We also saw a qualitative difference in the

antibody. We never published those data. But that's interesting.

If there were to be a persistent infection associated with a live agent, yes, I think you would see that.

For the rare cases of encephalitis that occur following vaccines, measles vaccines, the IOM safety committee determined the evidence was -- well, I had better be careful. I'm not certain. Most of us think it can occur. It's extremely rare. But most of the cases of encephalitis are coincidental. So when you study an individual, it's hard to know what's going on.

But my guess would be that you would see differences in the CSF. In one such publication from a Canadian study, it was done.

So maybe, but rarely. For the most part, some of the other serious adverse events -- the whole-cell DTP-associated encephalopathy probably wasn't antibody-mediated; it wasn't immunologically mediated.

I'm trying to think of some others. With the Guillain-Barre, nobody looked at the serum antibody quantitative titers back in 1976, but certainly we need to be studying that.

DR. CONSTANTINE-PATON: I have a question about

this decrease survival in girls. I was just looking more carefully at your slide. In the U.S., it drops to 8 percent over boys --

DR. HALSEY: Hold on a second. The survival figure is not from the U.S. The survival figure is a study from the Senegal that I showed.

DR. CONSTANTINE-PATON: So is there evidence that girls are more malnourished than boys in that country?

DR. HALSEY: No, there was no evidence that girls are more malnourished in the three countries with the increase. We looked for such evidence. There was no evidence in Haiti, where I did studies.

I believe there's something immunologically different about them.

DR. CONSTANTINE-PATON: These are nine-month-old girls.

DR. HALSEY: Those children were vaccinated between six and 11 months, six to nine months in Senegal and Guinea-Bissau and six to 11 months in Haiti.

DR. MARKERT: In patients who developed the encephalopathy or other neurologic complications, in recent studies, are investigators looking at tetramer-positive cells to see if there are more primed CD8-positive cells circulating?

DR. HALSEY: First of all, now we rarely see encephalitis/encephalopathy. After the acellular DTaP, I don't think that the evidence is there to say there is an increased risk following that vaccine.

Is that the vaccine you were associating it with or were you thinking about live viral vaccines?

DR. MARKERT: Live viral.

DR. HALSEY: There is a group that's collecting information. There are actually a couple of studies. The state of California has been running an encephalopathy/encephalitis study for many years. They have a panel of neurologists, immunologists that are collecting specimens and doing studies. They are looking at a number of different things. I know they are looking at differences in immune response.

The real problem here is that the vast majority of cases of encephalitis or encephalopathy that follow vaccines are due to other factors. They are coincidental. We see the background rate -- 1 in 100,000, 1 in 1 million per year. Some occur by chance alone. So it's very hard to get a very clean group where you have good evidence for the vaccine as the cause and then you can do studies on those. That's the difficulty in doing these studies of very rare events.

I don't know of any specific study looking at the specific issue that you mentioned.

DR. KOMAROFF: A related question to Betty's. Is there any association across vaccines between the frequency or variability of inflammatory symptoms and signs, on one hand, and inflammatory cytokine production, on the other?

DR. HALSEY: There is a great deal of variability with regard to the inflammatory signs. Many vaccines produce minimal. You see minimal physiologic changes, which we then assume means that there are minimal cytokine or other measures of inflammation. We do have a small number of studies -- one study in preemies that showed that there was an elevated CRP transiently following, I believe, DTaP in preemies who stayed in the nursery long enough to get that. There are some physiologic changes following vaccination.

The second part of your question I already forgot.

DR. KOMAROFF: Was there a correlation? When you see this variability in symptoms and signs, is it correlate with a variability in proinflammatory cytokine production?

DR. HALSEY: One study has been done -- at least one -- with smallpox vaccine. That's a study that I'm interested in and would like to see done with other

vaccines. We need that for some influenza and other related vaccines.

I didn't look specifically in the literature for all such studies, but I think there is a very small amount of data. It's not routinely measured in most studies.

But based especially on the cortisol and the other things that we are seeing, we can expect that there probably are some inflammatory markers that will be changed somewhat following many different vaccines. The timing of that will vary. Live vaccines will have a delay for the incubation period for the replication. The inactivated may be earlier.

But there are some that cause minimal physiologic changes. Hib conjugate vaccine by itself doesn't cause much. We get a little bit of local redness, but not much.

DR. NGUYEN: Dr. Halsey, is there a literature on the immune response among infants whose mothers received a similar vaccination during pregnancy -- specifically, seasonal influenza?

DR. HALSEY: Yes, there is some literature. There are studies on tetanus, IPV in particular. Getting a dose of tetanus toxoid increases the mother's antibody titer and it can decrease the response to the vaccine being given in infants starting at six weeks of age. It doesn't

inhibit enough to prevent a protective level of response.

With influenza, the most recent study, done by Mark Steinhoff and his colleagues in Bangladesh, showed that vaccinating mothers with influenza vaccine protected the infant against influenza. I'm pretty sure they have the blood specimens and have not yet analyzed them to look at the amount of antibody that may have been transferred. It's basically the passive antibody. They did not look at subsequently vaccinating those children.

But that study has stimulated a lot of interest. There are at least a half a dozen other studies going on looking at the potential benefits, practicality, and immunology of vaccinating women late in pregnancy and the effect on the infant and the infant's response to influenza vaccine. But the studies haven't been completed to date.

I mentioned IPV, tetanus. With IPV, there is some blunting of the response.

DR. CLAYTON: Thank you very much for that really rich talk.

We will now hear from Dr. William Banks, professor in the Departments of Internal Medicine, Geriatric Division, and Pharmacologic and Physiologic Science at St. Louis University School of Medicine.

Agenda Item: Antibodies, Vaccines,

UNEDITED VERBATIM TRANSCRIPT

Neuroinflammation, and the Blood-Brain Barrier

DR. BANKS: Thank you very much. It's a great pleasure to be here today.

My conflicts don't relate to anything I'll talk about today. They mostly relate to biotech companies, imaging for Alzheimer's disease, drug delivery for Alzheimer's disease, which does not include vaccination or that approach.

This focus slide, of course, is Louis Pasteur, *Vanity Fair*, circa 1887, "Men of the Day," one of the few non-British scientists that magazine illustrated. I thought he was a great poster child for us today, because not only did he do pioneering work in vaccination, but he also had to deal with problems of vaccinations and adverse effects, since many of his patients who received multiple injections with spinal cord homogenate from the rabbit had some problems.

I had some trepidation when I was first invited to talk about this, because about the only thing I really know about vaccine safety is that you are not supposed to recap the needle, which I think isn't what you meant. But when I saw the questions you were asking, I said, okay, I do know something about this. In fact, my lab has worked on some aspect of almost every one of these.

UNEDITED VERBATIM TRANSCRIPT

I'm going to use these not so much as an outline, but as talking points. Let's go through them quickly, so we all have a feel of where we are going to go. The questions posed:

- What normally crosses the blood-brain barrier?
- How does inflammation affect the BBB?
- Are there data that responses to infection or vaccine can alter the blood-brain barrier?
- What's the time course?
- Are there individuals with poor blood-brain barrier integrity? Who are they?
- What is the age after birth when antibodies and cytokines no longer penetrate the blood-brain barrier?

I read into this question, given that the neonatal or perinatal blood-brain barrier is immature and leaky, at what age do antibodies and cytokines stop leaking into the brain?

These are all great questions. So let's get started.

I would like to take you back again to the end of the 19th century, when the famous Paul Ehrlich, as a student in 1885, did the following study that we have replicated for you. That was to inject basic dyes into animals and look at their distribution. Here we have done this with

trypan blue. All the internal organs are stained bright blue, with the notable exception of the central nervous system.

I draw your attention to the structure on the far left. This is the inside of the cranium. There is nothing here but bone and a bit of dura mater. Look at how dark blue it is. If I had thrown up a heart or a kidney, it would just look like an amorphous black blob, there would be so much dye. Yet here, less than a millimeter away, in the brain, no staining.

It was eventually realized that there was something preventing the dye from getting from the blood into the brain, a hematoencephalic barrier -- as we now call it, the blood-brain barrier.

Unfortunately, many people carry around as an image of the blood-brain barrier something that looks like this. This is the helmet of a knight -- this rigid covering of the brain with some slits in it for things to leak in.

But this is a much better model of the blood-brain barrier. In fact, this is what many people think of as the blood-brain barrier, although, as we'll talk about later, there are some parallel barriers, too. This is the capillary bed of the brain. It's modified in three major

ways. One is that wherever the endothelial cell comes upon itself or another endothelial cell, it's cemented together with tight junctions. That prevents intercellular leaks. There are no fenestrae, or intracellular windows, in the cerebral vasculature. Also there is very little macropinocytosis. If you look really carefully, you can see a few little holes in places like that. There are pinocytotic vesicles. But if you compare the vasculature of the brain and central nervous system to, say, the heart, in which you see a massive amount of macropinocytosis, you can see that there is very little going on there.

So we put these all together and what we have is a barrier where essentially there is no ultrafiltrate produced. This is really significant. One way of measuring this is to ask, how much albumin is there in the cerebrospinal fluid compared to the blood? The answer is, for every 200 molecules of albumin that are in the circulation, there is one in the cerebrospinal fluid -- so half a percent.

At this point in the talk, I want to go in two different directions. I want to use this as a basis to consider two different aspects of questions. One would be false positives produced by Ehrlich's approach of using dye, and the second is the physiological implications of

having no ultrafiltrate produced. I'm going to go down the second pathway first, which will get us to what does cross the blood-brain barrier.

Consider that if you don't produce an ultrafiltrate, which is usually the way tissue beds are nourished, how does the central nervous system get its nourishment? How does it get all the things that it needs? After all, this is the most metabolically active tissue in the body.

The answer is, the blood-brain barrier is also the answer here, because once it has defined this rigid barrier, it then decides what things it lets cross. So there are transports from blood to brain, transport systems from brain to blood, and bidirectional systems.

So a modern understanding of the blood-brain barrier is, yes, it's a great barrier, but, in addition, it has extra-barrier roles as well. It plays a nutritional role. Essentially everything the central nervous system needs to grow and maintain itself has to be transported across the blood-brain barrier. There are transports for glucose, amino acids, free fatty acids, vitamins.

There is a homeostatic role for the blood-brain barrier, so that electrolytes are transported involved in pH balance, and the export of toxins produced either from

the brain or derived from the exterior.

The area that our lab has been most involved in is that of communication, because we now know that there are regulatory substances that are transported across the blood-brain barrier, both into and out of the CNS -- things like leptin, enkephalins, and cytokines, et cetera, et cetera.

So what normally crosses the blood-brain barrier? The first answer is whatever the brain needs. One way to think about this is that the blood-brain barrier is salve to the central nervous system and is there to service its needs. One way that it does this is by a host of transporters. Again, as we have already said, this includes glucose, amino acids, electrolytes, regulatory proteins, including cytokines, free fatty acids, nucleosides, antibodies, even cells. These are all very different kinds of mechanisms. We'll touch on some of these.

What's also important, I think, for this group is to realize that sometimes things go wrong and some of these systems are co-opted by viruses and bacteria, and many drugs are also dependent on some of these transports.

Besides the actual transport systems, there are three main other ways in which things are getting across:

- Membrane or passive diffusion. This is the way many drugs get across. We'll talk about that in just a moment.

- The so-called extracellular pathways, which are the residual leakage of the blood-brain barrier, which doesn't occur at the capillary bed, but through very specific areas.

- Adsorptive endocytosis and diapedesis. This is important particularly for glycoproteins, and therefore is the way that many cells traffic across the blood-brain barrier, that immune cells traffic across the blood-brain barrier. This is how HIV gets into the brain as free virus, as well as through the Trojan horse. We'll talk a little bit about that, too.

Let's go back to passive diffusion. Things that can meld with the cell membrane can diffuse right into the CNS. The important part here is that it's non-saturable, it's lipid-soluble -- the more lipid-soluble you are, the better you get in -- and also inversely related to the square root of the molecular weight.

Some of my favorite, favorite drugs get here -- for example, morphine and ethanol and nicotine. I submit to you that we would have a very boring world if the blood-brain barrier didn't allow some of these things in.

Here's what you get, this sigmoidal curve here. Please note that this is a log-log axis, and so the sharp center upward linear part is over-accentuated. At any rate, the point is that the more lipid-soluble you are, the more you get in. If you notice that circle in the upper left-hand corner, those are transport systems. Those are the things that get in by transport. If you are lucky enough to have a transport system across the blood-brain barrier, then you are getting in somewhere between 10 and 40 times more than you would if you were just based on lipid solubility.

This is an old slide. What we need now is a similar circle down in the right-hand corner for things that are transported in the brain-to-blood direction, substances that are exported out of the brain. Efflux systems are now a very, very important part of understanding the blood-brain barrier, particularly in the drug-delivery world, but also in other aspects as well.

These are some of my favorite efflux systems. Some of them are my favorite because we described them. Some of them are my favorite because they do such neat things. For example, consider the tobacco worm. How can this worm eat this toxin, nicotine? No other worm can do that. The answer is because it has developed a brain-to-

blood transport system for nicotine, and so it keeps it pumped out. I think that's pretty neat.

Lomotil -- I don't know if any of you have children. If you have had children, your children have had diarrhea. You probably used Lomotil to prevent that. Lomotil is an opiate, and what it really is doing is inducing opiate constipation. You could use morphine just as well to prevent the diarrhea. However, it would cause obtundation of the child, and therefore is not considered very good. So we use Lomotil instead.

But if Lomotil is an opiate, how come it doesn't cause CNS effects? The answer is because it's a very strong substrate for P-glycoprotein, an efflux system. If you give Lomotil to knock out animals that don't have Pgp, they have the same effects as if you give them morphine. Pgp, in particular, runs through -- unknowingly, for decades -- which drugs we use that have CNS effects and which ones don't.

This is a partial list of drugs that use Pgp, that are effluxed by Pgp. I have selected them because they represent such a diverse group and because many of these are very commonly used: digoxin, vinblastin, vincristin -- vincristin is -- the more avid the transport is, the less CNS effect -- peptides, verapamil, protease

inhibitors. Many of the reasons that HIV drugs don't really treat CNS or neural AIDS is because they are pumped out of the brain. Pgp is part of that. There are other systems for AZT, et cetera, et cetera. So this runs through a whole system of what we use. We are just now beginning to realize that we have to come to grips with this system in order to do drug development, et cetera, et cetera.

That, very quickly, is a flavor of not only what crosses, but why it crosses and what the major mechanisms controlling it are. Now I would like to go back and pick up the other stream of thought, and that is the false positives that can be induced by Ehrlich's dye study.

Why doesn't that basic dye cross the blood-brain barrier? The answer is because it tightly and avidly binds to albumin. If you give too much dye, you can saturate the albumin. You can have free, unbound dye, and that will cross the blood-brain barrier absolutely fine.

This is what happened, about 1927. There is this idea that the blood-brain barrier of the neonate just doesn't measure up, that it's somehow leaky or immature. This probably goes back to Behensen's study in 1927, when he gave large amounts of dyes, over several days, and then looked to see if there were barrier regions in the neonate.

Still, most of the regions showed barrier function, but somehow this was interpreted as the neonatal blood-brain barriers being leaky. Very strange, because in 1920, Wislock had done the studies appropriately and got the same results as in the adult.

In 1929, Stern did a review, as well as original data, and showed that if you give too much dye, you will get false positives of disruption.

But there's something very intriguing about an immature brain having an immature blood-brain barrier. As Barcroft said in a review, there's no reason why the brain of the embryo should require an environment of very great chemical constancy. I think the view then was, you have this amorphous, premature sort of developing brain, and so it doesn't need a very consistent environment.

I think we have the opposite view now, that the developing CNS is a highly regulated area and requires a lot of special circumstances. Yet we still have this idea that the blood-brain barrier is very primitive.

Norman Saunders, in Australia, has spent his life looking at the neonatal and perinatal blood-brain barrier, using very interesting models. Since he is in Australia, for example, he is able to use marsupials. Of course, you simply pluck the fetus from the pouch. So he has a very

easy model. And he has done other things as well. He points out that it's possible to demonstrate a barrier in the developing brain without injecting anything. The circulating plasma contains various proteins, and these can be visualized in tissue sections by immunohistochemistry.

So we have all kinds of methods that we have looked at the neonatal blood-brain barrier with. They all tell us the same story. Pretty much, the neonatal blood-brain barrier in regards to the serum proteins is just as mature and just as tight as anything the normal adult has. This has become a real issue. I would like to just share with you one experiment that we have done with Bill Sly. There is a group of diseases called mucopolysaccharidoses. These children are born without certain enzymes, and so they develop GAGs in the peripheral tissues and in the brain. Of course, we can produce huge amounts of enzyme these days and treat these patients with it. Regardless of whether we treat the patients when they are young or older -- or the knockout mice when they are young or older -- we can clear all the GAGs from the peripheral tissues. Unfortunately, of course, these enzymes don't cross the blood-brain barrier, and so there is no recovery in the brain.

But interestingly, if you treat neonates -- one

or two days in the knockout mice, very early in the children -- you can get recovery in the brain. So the question is, why do you get recovery in the brain of the neonate? Is it because there's a leaky neonatal blood-brain barrier or could there be a saturable transport system that disappears with maturation? It turns out that it's the latter.

Here's the data to look at this. The main reason for showing you this is to illustrate that there is indeed a barrier to serum proteins in the neonate. If we look at the red line, this is the rate at which this enzyme is crossing in a two-day-old mouse pup. We see it's crossing at a pretty good rate. The K_i is a measure of the influx rate.

The green is the albumin rate. You see there is essentially no albumin crossing. The enzyme is five times bigger than the albumin, so it's not leaking in. This is co-injected. The albumin is labeled in one way and the enzyme the other way. So the animals are getting both of these substances. Each animal is its own control.

This is actually enzyme in the adult, but the albumin curve looks exactly the same. You see that albumin injected into the neonate, albumin injected into the adult -- there's no transport at all.

This isn't to say that the neonate blood-brain barrier is exactly the same as the adult. It is not. It does have a lot of subtle differences, and this is an important one. This was published by Peruzzo. What happens is, there are areas of the brain, here illustrated by the ME, which stands for the median eminence -- these are proper parts of the central nervous system. But the capillaries that go through them don't participate in tight junctions, et cetera, et cetera. They look like peripheral vessels. It's very leaky.

What happens in the adult is that you develop right here at the junction between the median eminence and the arcuate nucleus, a part of the brain that does have tight junctions, another arm of the blood-brain barrier, a tanycytic barrier. It hasn't been studied all that well. But the neonate, when it's born, does not have the tanycytic barrier. What this means is, if you inject a neonate with monosodium glutamate, it can enter the median eminence, diffuse over to the arcuate nucleus, destroy it, and these animals become obese, whereas if you give the monosodium glutamate to an adult, the arcuate nucleus is preserved. So think about that before you take your kid out to eat in a Chinese restaurant. Ask them for non-monosodium glutamate food.

The other aspect that's interesting -- so you might say, okay, it looks like there are aspects that are immature. But I submit to you that it's not so much immature as different. If we look here, this is the adult. If we look at this ependymal lining between the cerebrospinal fluid and brain proper, there are no tight junctions here. Things can diffuse from the CSF into brain tissue. In the neonate, though, there is a barrier here and things cannot diffuse easily from the CSF into adjacent tissue.

So again, it's different, for reasons we don't really understand. It makes sense that it's different, because, as I said, the blood-brain barrier is trying to serve the brain. The developing brain is different from the adult brain, and so maybe it's not surprising that there are all these subtle differences.

One concept is, the blood-brain barrier adapts to the changing needs of the CNS, with maturation, aging, and, as best it can, disease. The flip side of this coin is that when the blood-brain barrier does not adapt or there are problems at the level of the blood-brain barrier, we can develop disease.

Here are three examples that are very well researched right now:

UNEDITED VERBATIM TRANSCRIPT

- Obesity has a resistance to leptin. Leptin is produced in fat, crosses the blood-brain barrier, acts at the arcuate nucleus to decrease feeding. In obesity, there is a resistance to leptin. The first level at which leptin resistance develops is a defect in the ability of the blood-brain barrier to transport leptin. So in some ways, obesity could be viewed as a disease of the blood-brain barrier.

- Alzheimer's disease, which many people believe is caused by an accumulation of amyloid beta protein. Zlokovic and others have shown that there is a failure of the ability of the blood-brain barrier to pump A beta out of the brain, because there is a defect in the function of LRP, which is the efflux system for that. This defect even occurs in animal models of Alzheimer's disease, either the transgenic overexpressing A beta or the SAMP8 natural mutations.

- Then there's De Vivo's disease, which is in families that express only 50 percent of the level of the Glut-1, the transporter responsible for transporting glucose into brain. They only express at 50 percent normal. They have familial retardation, epilepsy, and some other CNS problems.

So again we realize that the blood-brain barrier

itself can be the seat of disease and a therapeutic target and that maladaptions are a problem.

This answers partly the question, what is the age after birth when antibodies and cytokines no longer penetrate the blood-brain barrier? We have to re-ask this question. It's not that the blood-brain barrier is immature. But it begs the question: Do antibodies and do cytokines enter the brain? Do they cross the blood-brain barrier? So now let's turn to that question. The answers for antibodies and cytokines are very, very different answers.

Here's a paper that we published in 1989 -- very controversial when we did it. What we have done is, we have radioactively labeled albumin with ^{125}I and the cytokine interleukin-1 alpha with ^{131}I and co-injected them into the same animals. This data set is from the same animals, each animal as its own control. If you go out long enough, you can detect the rate of entry of albumin into the brain. Here we see that we can barely detect a slope in the albumin line. It's about 10^{-5} , 10^{-6} mL/g/min. The entry of interleukin-1 alpha is 40 times faster than that. Even though interleukin is only about one-fourth smaller than the albumin molecule. So clearly this could not be leakage. It must be a transport system.

UNEDITED VERBATIM TRANSCRIPT

Indeed, that's what we show formally here. We have repeated the study, in which we injected either radioactive interleukin or radioactive interleukin with an excess of unlabeled, and we see that we can begin to inhibit the transport system.

Here is combined data for the albumin control, with and without the interleukin. You can see that since the albumin line doesn't change, there is no disruption of the blood-brain barrier by these doses of interleukin. Of course, a disruption would increase the rate at which interleukin would enter, not decrease it. But you kind of want to make things easy for the referee -- not have to make them think too much. So we made sure to include the albumin control.

Interspecies differences in the transport of cytokines: Here we have murine interleukin-1 alpha -- much, much faster. Of course, these studies are done in mice. Mice transport mouse interleukin-1 alpha much faster than they transport human interleukin-1 alpha. So there is an animal species difference.

There is also a chemical species difference. This transporter prefers murine interleukin-1 alpha to 1 beta. So there is a species difference that way, too.

Another species difference is that the

transporter is inhibited by unlabeled interleukin-1 alpha, but not TNF, unlabeled TNF.

So we can begin to look at the question of which cytokines are transported, whether there are families of transporters, whether there are independent transporters, by simply building up grids like this, where we inject radioactively labeled cytokines and unlabeled cytokines. We see that there is a family of cytokines that are transported for interleukin-1 alpha. IL-2, for example, is not transported. IL-6 is, but not inhibited by the IL-1 system or the TNF system. So we build up all of these sorts of systems.

There was just a paper in *British Pharmacology* that just came out in epress. Nancy Rothwell's group has looked *in vitro* and confirmed our *in vivo* data, dating back some 20 years.

Interleukin-1 alpha, interleukin-1 beta -- these systems are the tip of the iceberg. There are many saturable transport systems for different families of cytokines, as well as specific cytokines that are clearly not transported.

We now know that the transport systems for cytokines are important. This paper was published a couple of years ago by F.T. Crews. It shows how some of these

systems may work. Crews and others have noted that if you treat animals with lipopolysaccharide, you can induce a death in dopamine cells in the substantia nigra. Here's how he figured out it worked: The LPS releases TNF from peripheral sources. A portion of that TNF crosses the blood-brain barrier and interacts with the microglia to release brain levels of TNF, which then kill off the neurons.

We showed a variation on this theme some eight years ago, when we looked at the effect of interleukin-1 alpha induction on cognitive impairment, which is one of the parts of the sickness behavior that interleukin-1 alpha induces. We looked at it by giving blocking antibodies, the logic that we knew that most interleukin-1 alpha enters the brain at a structure called the posterior division of the septum, and so we gave antibodies that either were specific for human interleukin-1 alpha or were specific for murine interleukin-1 alpha. We infused human interleukin-1 alpha with the logic that if it were the interleukin crossing, the interleukin-1 alpha, human, would block the effect. Indeed, that's what we found, as opposed to the murine blocking.

So this is a little bit more pharmacologically oriented mechanism showing much the same thing that Crews

found a few years later: Cytokines crossing the blood-brain barrier do indeed interact in CNS tissues to affect, now we know, both neurodegenerative processes and cognitive processes.

Do antibodies cross the blood-brain barrier?

I really like this topic. I go to meetings, and it seems that there is more information and more discussion and more reviews about this very topic than there is primary literature. We decided a few years ago to actually look at this. We were interested in the question from the amyloid beta protein/Alzheimer's disease sort of view. So we co-injected once again albumin labeled with 125 and antibody labeled with ¹³¹I -- again, an antibody directed at A beta.

This is an IgG molecule. What we find is that, for about the first hour and an hour, although the antibody level is a little bit lower, there's no statistical difference between these two lines. We figure the antibody is leaking in through the same pathways that are used by albumin.

Notice that these are really low rates. These are 10^{-5} mL/g/min. But, yes, some antibody is getting in.

The way that albumin gets in is through a series of pathways called extracellular pathways. They were

called "functional leaks" and then Broadwell (phonetic) studied them much more formally in the 1980s and 1990s and reified them as the "extracellular pathways." These are not at the capillary bed, interestingly. The blood-brain barrier can totally keep proteins out completely, but it has decided, it seems, at the last minute, to open up these residual pathways, these backdoors, to let a little bit of serum proteins in. These were defined using albumin and horseradish peroxidase at the EM level, the light microscopy level. Now we know that antibody and erythropoietin cross.

We are beginning to wonder if these could be used therapeutically. Certainly for antibody delivery, that's a central question. Just as the lipid solubility has its characteristics for its substances which enter, the ideal substance here would have a very long half-life, it would be enzymatically resistant, it would have a small whole-body volume of distribution -- in other words, it stays in the blood and it lasts forever in the blood, so it's presented to the blood-brain barrier constantly, so that these very slow pathways can eventually accumulate in the CNS -- and it must have a high CNS potency. You are not going to get much in, so you have to have something that really is pretty potent.

UNEDITED VERBATIM TRANSCRIPT

But then, if we keep looking and we go to hours -- and look how long we study this -- we see that there is a difference in the rate at which the antibody gets in compared to the albumin. This is probably due to the efflux that has long been suspected for IgG molecules, mediated through the neonatal Fc receptor.

This has been around for a long time. There are maybe only half a dozen papers, but they are scattered. There is some seminal work by Partridge (phonetic) in 2002, showing, yes, that that receptor truly is on the blood-brain barrier, and a few years ago by Zlokovic's group showing, yes, it's functionally exporting -- although, interestingly, Garge(?) has just published a paper, only available in epress, where he raises a doubt, whether the Fc receptor is really working on the antibody. But I think when the dust settles, it will be shown that certainly there is efflux of IgG1 and it is probably largely mediated by the FcRn.

I don't know what this means. Is this good news or is this bad news? It could be bad news. Obviously, it limits the life expectancy of the antibody in the brain. Here we show the percent of an injected dose of antibody that gets in the brain. You see that it peaks in one hour and then, because of the efflux, it's sort of transported

out -- rather slowly, but deliberately -- out of the CNS. So it has limited exposure time. But maybe this is good, because maybe it's in there long enough to grab hold of the naughty thing that you want to get out of the brain and then it gets effluxed and dragged out. That's what Zlokovic thinks happens with A beta. So I think the jury is still out.

That was IgG. If you want to talk about Fab, Fc -- much less studied. But they have different, probably, characteristics. For example, IgM has a very different characteristic. This may be the only paper on IgM. It's something we did with Steinitz (phonetic), published a couple of years ago -- again, an antibody directed against A beta. We are looking at the SAMP8 mouse, which, with aging, accumulates A beta. The idea is that if the antibody is crossing, it will lock onto the A beta, and so accumulation will increase.

These are antibody-to-albumin ratios. You really expect, if that's working, to have a ratio of greater than 1 -- that is, the antibodies accumulating better than the albumin. We see that for one of the antibodies, the HyL5 it's not very exciting.

But for the L11.3, it was exciting. There was indeed some increased uptake and retention. Indeed, that

antibody, when injected intravenously, had effects on acquisition in this model of Alzheimer's disease. Enough was getting in to improve learning. Memory did not make statistical significance, but learning was improved almost to the level of the normal young mouse.

How does inflammation affect the blood-brain barrier?

We need to introduce some new concepts. From a review by Ed Newolt(?) last year, we need to talk about the neurovascular unit. This is John Steinbeck [sic] come to cell biology: No cell is an island. What we have to realize is that the brain endothelial cell or the choroid plexus -- whatever aspect of blood-brain barrier you want to talk about -- is constantly interacting with the other cells in the central nervous system of nearest proximity, which are the astrocytes, the neurons, the pericytes, the microglia, as well as with the circulating substances, including circulating immune cells. The brain endothelial cell can almost be thought about in terms of immune cell. It is activated at some level normally, and then, when insulted or challenged, that level of activation can increase.

The other concept I want to introduce, before we go a little bit further in this idea, is the functions of

the blood-brain barrier. We talked about the barrier function, the lipid solubility, and the transport functions. It also is an enzymatic barrier, which can be regulated, and it's a secretory cell body. In other words, the brain endothelial cells and the choroid plexus cells are themselves secreting cytokines, nitric oxide, substance P, prostaglandins.

So what we have here is a diagram of all the ways the blood-brain barrier interacts with the neuroimmune system, if you will. We have in the circle various immune effectors. Think about these as either a very diffuse way or whatever your favorite immune effector is -- a virus, lipopolysaccharide, cytokine, whatever. A shows that these things can induce the blood-brain barrier to secrete cytokines or, B, the other immune-related substances -- nitric oxide, substance P, prostaglandins. C shows that cytokines themselves can cross the blood-brain barrier, and once there, they can then cause cells within the CNS either to release other neuroimmune substances or other cytokines. D shows that these substances can alter other aspects of the blood-brain barrier -- their receptors, their transporters, whatever. The other part of D, with the immune cells, shows that amongst those things whose transport can be altered are immune cells, and once immune

cells are in the CNS, of course, then it's also possible to secrete new immune substances.

So we have a very complex, very elegant, very interactive thing. I think one of the big challenges in neuroimmunology is to realize that it's probably not a single thing and a single disease, but many things are happening at the same time by which the neuroimmune system is communicating across the blood-brain barrier. There are, of course, other pathways also, like the vagus, afferent nerves. So probably all these things are going on and interacting at the same time.

If we just consider one effector, lipopolysaccharide, it has all these functions on the blood-brain barrier:

- Disruption.
- Altered adsorptive endocytosis, which, for example, means that you can you get enhanced transport of viruses across the blood-brain barrier.
- Increased diapedesis, which means that immune cell trafficking across the BBB is altered. That has effects for immune surveillance, multiple sclerosis, Trojan horse mechanism of HIV entry.
- Altered transport systems. I think this is the most fascinating part, because this is really the bread and

butter of the blood-brain barrier -- transporting things in and out of the brain. Insulin transport across the blood-brain barrier is altered. Pgp is altered. That means that if you have a person with an ongoing neuroimmune sort of activation, the drugs which are getting into the brain and out of the brain are going to be altered. Increases in RAGE and decreases in LRP -- we'll talk about what that means in a moment.

- Of course, the blood-brain barrier is secreting cytokines.

These various effects of LPS, we know, are not mediated monolithically. For example, here we have just compared HIV transport versus blood-brain barrier disruption. One is dependent on MAP kinase 44/42; the other one is not. But it's dependent on MAP p38; the other one is not. We are finding all kinds of examples. The cell biology by which LPS does its various things is mediated by different cellular pathways.

So if you are looking for an area to get into, come work on neuroimmunology and the blood-brain barrier. Lots of great questions.

Here is one implication. Amyloid beta protein is thought to relate to Alzheimer's disease. A beta is transported from blood to brain by RAGE. That's the

receptor for advanced glycation end products. It's transported out of the brain by the transporter LRP-1.

A recent study that we did showed that if we give LPS, it interacts and releases unknown factors into the blood. These blood-borne factors increase RAGE, which will increase the amount of amyloid beta protein into the brain. At the same time, it introduces prostaglandins, which work at the level of the blood-brain barrier to decrease LRP function, which will further increase A beta because it's no longer being influxed.

So we have LPS increasing the blood-to-brain transport of circulating A beta and decreasing the brain-to-blood efflux of A beta. This provides a mechanism by which inflammation could promote Alzheimer's disease, by altering blood-brain barrier transport of A beta.

Again, many substances are released from the blood-brain barrier. We have sort of talked about this already.

What's one of the neat things is the models that we can use for A beta. We have a very nice *in vitro* model. What we need to realize is that the blood-brain barrier, which, if you think about it, is a capillary bed, a tube going through the brain -- the luminal, or blood-facing, side of that is very different from the brain-facing, or

abluminal, side of the blood-brain barriers -- different transporters, even different lipid molecules making up the cell membrane. If we grow these endothelial cells in culture as monolayers, they polarize and they maintain their luminal versus abluminal differences often, and so we can study brain-to-blood/blood-to-brain transports.

Here we have done this, looking at cytokine release, just looking at IL-6 as the most prominent example. IL-6 is released both into the brain side of the *in vitro* culture and into the blood side, but the blood side secretion is favored. There's 10 times more being released into the blood side.

If we treat the *in vitro* cultures by adding LPS to the blood side, we can increase IL-6 secretion by fourfold. But if we add it to the brain side, we increase by eightfold, which is highly statistically different from the luminal side. So we have polarization of the neuroimmune axis mediated by the blood-brain barrier, not only in terms of constitutive release, but also in the way that it will respond to neuromodulators.

Are there data that responses to infection and vaccine can alter the blood-brain barrier?

I can't find very good examples. This is work that was done by Mabondzo about four or five years ago

looking at the blood-brain barrier secretion of endothelin-1. Many people have related that the higher your CSF levels of endothelin-1, the higher degree you have HIV encephalopathy. The question was, does HIV release endothelin-1 from the blood-brain barrier? He found that, yes, again using this monolayer system, HIV increased mRNA expression by 20-fold in the endothelial cell and secretion into the luminal side by fivefold. He could replicate this with gp120 only, which is the viral coat of HIV, but not the non-glycosylated aspects. So this seems to be relating to that.

Are there individuals with poor blood-brain barrier integrity?

This is a very fascinating question. Obviously people with things like multiple sclerosis/EAE, neurotrauma, stroke, and hypertensive encephalopathy. Alzheimer's has gone back and forth, whether there are changes, disruptions. There are certainly not large-scale disruptions. There may be micropunctate kinds of problems. No one knows.

When I was a student first getting interested in blood-brain barrier, everything had a disrupted blood-brain barrier, supposedly -- schizophrenia, for example, things like that. Those have been largely shown to have normal

blood-brain barriers.

But now we are back to the old idea again. We now know there are many diseases in which lipopolysaccharides are actually circulating in the blood because of bacterial translocation -- GI AIDS, periodontal disease, even obesity. Diabetes -- there have now been studies conducted to show that in streptozotocin-induced diabetes, after a relatively long period of time, blood-brain barrier disruptions start to occur. Even in chronic pain syndrome, there are disruptions. These are mediated through the neuroimmune axes, interestingly.

Even with blunt trauma, the disruption that occurs is very strange. Here we have done an experiment where we have cut the lumbar spine between L2 and L3 -- just a blunt trauma. What we see is, essentially, immediately after cutting, no disruption. There is some bleeding, but that stops. There is really no disruption or increased leakiness, until quite some time further out. This has been shown again and again with various problems and disruptions to the blood-brain barrier, traumatic disruptions. There are ways of opening and closing of the blood-brain barrier that really are temporally remote from the injury and have long been thought to, again, be mediated through the neuroimmune processes.

UNEDITED VERBATIM TRANSCRIPT

If we look in these same animals at what happens to a transporter for PACAP, which is a peptide that's related to neuroimmune conditions, we see a quite different picture. We see quite often that there are a whole bunch of arrows here; there are a whole lot of things going on.

I want to show you this, where we compare the albumin and the PACAP. These are the same animals -- again, the same trick of giving the albumin and the PACAP to the same animals. Whereas there is little and late disruption at the blood-brain barrier, this transporter first begins to be decreased in the brain almost immediately after the lesion, and as we progress down through the spinal cord, that decrease becomes less and less evident and a temporally later increase in transport becomes more and more evident. So a very complex sort of interaction that's going on, the details of which we don't understand.

I think I have run through most of the questions now, to some extent, and tried to give you an idea of where we are in this field. Rather than give you a summary slide of a lot of conclusions, I just want to leave you with this view, sort of an architectural view, of the neurovascular unit, and remind you that what this illustrates is, first of all, that the blood-brain barrier is not so much a

blood-brain barrier. If we were to discover it and reify it at the beginning of the 21st century rather than the end of the 19th century, I think we would call it the "blood-brain interface." It's interacting with all the cells in the CNS, in the periphery. It's doing many things besides acting as a barrier -- transporter function, enzymatic function, and secretory function -- and helping to regulate the way that the peripheral tissues and the CNS interact, and can be responsible for producing diseases.

On that note, I think I'll end. Thank you.

DR. CLAYTON: Thank you very much. Paige?

Agenda Item: Discussion

DR. LAWRENCE: I just wanted to follow up on your elegant experiments where you were looking at whether antibodies could cross this interface. I have two questions.

One is, for those studies where you are using radioactive antibodies, just to help me interpret them, were those physiological concentrations of antibody or super-high, getting to your point that trypan blue -- you could push false positives?

DR. BANKS: Neither. The advantage of using radioactivity is that we can inject very low amounts of material, so we feel that we are not perturbing or

interacting with the system. We can label these things, particularly things like antibodies and big proteins. They label so easily. We get high specific activity. So what we hope we are doing is just sort of blending in with the crowd of anything else that is there, and hopefully studying physiological levels.

DR. LAWRENCE: Switching gears, but just for me -- I'm an immunologist -- I want to understand what you are saying about the neonatal blood-brain barrier versus the adult. It's wrong to think that it's leaky in the neonatal period; it's just biochemically different?

DR. BANKS: Absolutely. If you ask the question, "What's the characteristic of the neonatal or perinatal blood-brain barrier to serum albumins," the answer is that it's just as tight as the adult.

Now, there are always caveats and exceptions in science. The one caveat is that right at the edge, where angiogenesis is occurring, the tight junctions haven't formed yet. The thing is in progress. Yes, that's a little leaky. But in the formed vasculature, to serum proteins, it's just as tight as in the adult. If you begin to think about our modern view of what's going on in the CNS, we would need that. Otherwise, we are getting toxins and things coming in.

However, the specific characteristics might be different. This amino acid might get in faster and that one might be slower. That probably relates because the developing CNS might need more of this amino acid for neurotransmitter development or whatever.

So again, the key to looking at the healthy blood-brain barrier is that it's trying to serve the brain, to protect it with barrier function and then to serve its needs with transporters or whatever it takes.

DR. CLAYTON: Les.

DR. WEINER: Very elegant presentation.

There are three parts to my question. The first is, after the polio vaccine was developed in the late 1960s, people showed that there were antibodies in the spinal fluid of normal people. They tapped volunteers, which happened to have been medical students, and they found that it was about 1 to 200 to 1 to 400 of IgG antibody in the spinal fluid after an oral polio vaccine. Nobody knows how long those lasted. Basically, that was done, I think, about six weeks after.

I was in the institution that did that, so that's how I know --

DR. BANKS: Were you one of the medical students that volunteered?

DR. WEINER: No, I was not. I was a medical student, but I was not a volunteer.

Anyway, the second point is that there are diseases which are antibody-driven, like Sydenham's chorea, stiff-man syndrome, and several others, where the degree of disease, clinical disease, from a peripheral stimulus is really related to the level and avidity of the antibody. So the question really is, is that a breakdown of the blood-brain barrier or is that simply the same system that gave you a 1-to-200 or 1-to-400 -- as you had low levels of immunoglobulin in the spinal fluid, which is normally there.

So that's the second question.

The third question is, activated T cells and B cells cross the blood-brain barrier. Is that a disruption of the blood-brain barrier or is that simply the ability of those cells to stop at an endothelial cell and make their way, by a variety of reasons, into the nervous system?

Those are the three questions.

DR. BANKS: The immune cell is probably the easiest to answer. You are absolutely right. There is an increasing belief that normally immunosurveillance is going on. Much of this work, related particularly to the basic science, to looking at EA-induced sorts of conditions --

ordinarily, there are probably not enough immune cells to capture these by the methods that are used. But there is an increased sense that under normal physiological conditions, there is trafficking going on there.

Whether in the normal condition or in the diseased condition, it is not a disruption of the blood-brain barrier. If you recall the capillary bed I showed you, there was this little smiley cell there. That was a red cell. The diameter of the capillary is about 5 to 6 microns. Of course, an immune cell is about 10. So it's architecturally impossible that you have this little opening of the tight junction and the immune cell goes in. Instead, what goes on is, just down from the capillary at the post-venule level, which participates in tight junctions -- arterioles and venules, endothelial cells, are also participating with tight junctions and such -- a very elegant dance goes on called diapedesis. It's controversial, if the immune cell goes between cells through the tight junctions by inducing dissolution or if it goes through a given endothelial cell, like a donut. Probably both are true. It probably depends on which cell you are talking about, a lymphocyte versus a macrophage. But it's this very elegant diapedesis. The immune cell is using the same trick that it used to get out of the high

epithelium back at the bone marrow.

So no, it's not blood-brain barrier disruption. It's a very elegant neuroimmune communication. It's known that cytokines are involved and all that kind of thing.

The second question, in terms of the origin of antibodies in the CNS: I think it's very possible that antibodies can be entering discrete blood-brain barrier disruptions and causing havoc. To the extent that that gets translated into CSF levels, though, it may be a little tricky. There is not very good diffusion, once a substance is in a brain tissue, to adjoining brain levels, because the only thing really pushing it is Brownian motion and metabolic production of water. These things are a little bit slow, and the antibody is being degraded along the way. So it's tricky to use CSF levels to see what's going on in brain interstitial fluid. But it's certainly a possibility.

I don't think we really understand all the mechanisms by which antibodies are getting into the brain. Remember, for decades, this was the hallmark of the blood-brain barrier: The brain is an immunoprivileged area, antibodies don't really get in, and even though we find them in CSF, it's probably an artifact. That used to be a lot of thinking. I think we have to relook at

physiological and pathophysiological mechanisms.

Your first question was again?

DR. WEINER: That was just the idea that there is antibody after a vaccination, and we don't know how long it lasts. But clearly most of the central nervous system adverse effects are acute and short-lived. So the question is, is that diffusion of antibodies -- whatever their antigen target is, is not part of the question -- is it a diffusion of those antibodies or is this simply the same phenomenon that we saw with the polio vaccine, where the antibody entered, we don't know how long it lasted, but --

DR. BANKS: And we have a third possibility now, now that we understand that immune cells do cross the blood-brain barrier, even under normal conditions. It may be that immune cells are coming in and secreting their antibodies within the CNS. So I think we have, probably, three major candidates that are possibilities. The mix there -- we'll have to get some animal models and we'll have to look at these issues before we can really start sorting it out. I suspect the answer will vary, depending on the pathophysiology and the condition.

DR. CONSTANTINE-PATON: I have two questions. One is a quick one.

In your *in vitro* model system, I had always

thought that the astrocytes were very important in setting up a lot of the blood-brain barrier.

DR. BANKS: They are, yes. If you co-culture with astrocytes or with pericytes, both of these are a very important part of the crosstalk with the blood-brain barrier. You will get tighter junctions.

That doesn't necessarily mean that you need to always include them in your monolayer. It depends on the question. If you want to look at, for example, secretory questions, maybe you had better leave them out, because you really don't need super-tight junctions to ask those kinds of questions, because then you are going to get confused. Maybe the cytokines are coming from the astrocyte or the pericyte or something like that.

DR. CONSTANTINE-PATON: The other question just came up as you mentioned that while the blood-brain barrier is forming, it's permeable.

DR. BANKS: At the growth cone, yes.

DR. CONSTANTINE-PATON: It brought to mind the fact that there is a whole series of experiments, mostly done by Greenow(?) where he shows that an enriched environment -- what that does, before it does anything neural, is that it increases massively the vascularization. At times when there is major new neuropil forming -- it's

not new neurons at that stage; it's mostly new neuropil -- you would be most susceptible. That brought to mind the fact that adolescent diseases, like schizophrenia, tend to show up after puberty, which is, of course, when the frontal cortex neuropil is really enlarging.

I was wondering if that has ever come up in any of your --

DR. BANKS: I haven't thought of that. That's a very interesting concept. I'll have some fun with that idea.

DR. CLAYTON: Betty.

DR. DIAMOND: I was wondering if there are any data on what fever or seizures do to any functions of the blood-brain barrier, not just the integrity of the blood-brain barrier.

DR. BANKS: Seizures clearly disrupt the blood-brain barrier. Seizures probably alter P-glycoprotein activity, which is really pretty bad, and probably increase it so that there is increased Pgp efflux. That's not good. Almost every anti-seizure medication we have, with the exception of valproic acid, is a Pgp substrate. I'm sure there are people here who know more than I, but I understand that if you get people with status epilepticus, people who have uncontrolled seizures, it becomes

increasingly more difficult to control those seizures, to get ahead of the situation.

This may be one of the explanations for that -- increased seizures, upregulation of Pgp, and therefore ever more difficult to get effective levels of drug into the brain.

Fever is actually very interesting in that it's part of the sickness behavior that's induced by interleukin-1 alpha. The way that interleukin-1 alpha induces fever is to act at receptors, brain endothelial cells, and induce prostaglandin secretions that then induce the fever.

I don't know of any studies specifically looking at body temperature altering blood-brain barrier aspects.

DR. MARKERT: The first question is -- am I thinking about this right? -- in terms of T cells entering the brain, would it not be predominantly primed T cells? For them to then cause a problem, it would have to be primed to a peptide MHC that then they would encounter in the CNS. My understanding is that naïve T cells can't get primed in the CNS, either because of lack of dendritic cells or that they just don't get primed.

DR. BANKS: Probably. I don't think the experiments have rigorously been done to address all those

aspects. I think what we understand now is that there is an interaction of molecules, that there is a whole series of processes that go on, like capture and slowing of the immune cell, because it's going through the circulation, so it has to be captured and slowed. Some people feel that that's not necessarily leading to the next step of diapedesis, that perhaps just having the immune cell and the endothelial cell attached allows these to have a paracrine kind of communication that may be totally unrelated to further transport. So there are those aspects, too.

But, yes, I think the key, to sort of be brief, is that you have to have an activation not only of the immune cell, but also the blood-brain barrier. It's like speed dating: They both have to be willing to have this occur. Very fast speed dating, about 1 mL per minute.

DR. MARKERT: So if there is some reaction going on in the periphery, be it a vaccine or an infection, and you have increased levels of TNF, interferon gamma, and you have primed these T cells, the TNF and interferon gamma -- the effect they have on the endothelial cells for expression of MHC molecules and also just for their transport, are those physiologic concentrations? I do believe these can go sky-high, correct?

DR. BANKS: I think you have to look at the BBB as always a little bit activated and always kind of in the mode, so to speak, but now you can turn up the volume to various degrees. I suspect the immune cells, by and large, will be like that.

Interleukin-1 alpha is probably also involved. Kwon(?) did some beautiful experiments where he injected IL-1 into brain tissue and looked a few hours later, and all these immune cells came flooding into that area. So there is also some way that something going on in the CNS can call in these aspects.

I'm not sure that quite got to all the aspects of the question.

DR. MARKERT: Real quick, is LPS a substrate for P-glycoprotein?

DR. BANKS: No, it doesn't look like it. Unpublished data -- it doesn't look like it.

DR. WEINER: I might just add that if you activate endothelial cells, they make an enormous amount of MCT and other chemokines. It amplifies the whole system with very little inducement of MHC expression in endothelial cells.

DR. BANKS: And there may be a mechanism, according to a couple of papers by Roet(?), that certain

cytokines -- I think IL-8 is what he did -- injected it into the CNS, and it showed up on the luminal surface of the endothelial cell. If that's true -- and it looked pretty well done --

DR. WEINER: And endothelial cells *in vitro* make a ton of IL-8 as well.

DR. BANKS: So it's like they are able to put out this calling card, "This is what's going on in the CNS."

DR. SAMPSON: I enjoyed your lecture. I have two questions, one about the Pgp efflux system. In there you list dexamethasone as something that's effluxed rapidly. Yet when we give steroids, we see some individuals who almost get psychotic when they receive it. Does that suggest there are polymorphisms in this system? How do you explain that?

DR. BANKS: There are tremendous polymorphisms. In fact, there is great pharmacogenomics. I think it's 30 percent of people overexpress Pgp and 25 percent underexpress it, compared to the normal population. The overexpression has been suggested to coincide with the 30 percent of epileptic patients that are resistant to epileptic drugs and the 30 percent of patients that don't respond very well in terms of neural AIDS, because all those are substrates for Pgp.

The other concept that this is really critical for is that even though something is a Pgp ligand, then you have to ask, how strong of a ligand? Although, as I told you, Lomotil is a very strong ligand, so is morphine -- just not as strong. The question then becomes, if indeed Pgp is upregulated by things such as neuroimmune inflammation, we may be shifting that completely. For example, if Pgp becomes more active, morphine, which is a weak ligand, might now be pumped out even more so and might have less effect on pain than we might be expecting in our patients. Or you can argue the other way, of course.

There are a lot of implications when you start talking about either pharmacogenomics or just the neuroinflammatory aspects of Pgp.

DR. SAMPSON: The other question is related to tight junctions. I don't know much about them in the brain --

DR. BANKS: No one does.

DR. SAMPSON: Okay. But in the GI tract, there is certainly a lot of interest in a variety of the proteins that make up the tight junction. There are certainly effects of immune cells on the composition of these tight junctions and their effectiveness. For example, even in an allergic reaction, you can generate increased leakiness

because of alteration of these tight-junction proteins. Do people know anything about that in the brain?

DR. BANKS: For about the last five or six years, people have truly started looking at tight junctions in brain endothelial cells. Prior to that, much of what we knew or thought we knew about tight junctions came from studies in the GI tract. That was our forerunner.

Some of this data goes to grants I have reviewed, but there is definitely clear data now that tight junctions are altered by neuroimmune events, including the trafficking. What's interesting is that we are now going to probably enter this era where many of the cell biological aspects that affect tight junctions, like actin and that kind of thing, intimately involved in the transcytotic mechanisms -- I didn't get to talk much about that, although much of what I implied about some transporters and diapedesis is involved in the vesicular transport. Even though macropinocytosis is decreased, many other vesicular-related systems are very important to the way the blood-brain barrier operates and even, in some cases, dominate blood-brain barrier, quote/unquote, disruption. It's often an increased vesicular transport rather than the so-called paracellular transport of the tight junctions.

So that might even be more important for understanding disease and disruption than tight junctions. It looks like much of that pathophysiology, much of the cell biology, may be common to those two processes. That's really in its infancy.

DR. CLAYTON: Thank you very much. I have to say, this is about the richest series of talks I have heard in a while.

Our next speaker is Dr. Bruce Cohen. He is chief of the Section of Pediatric Neurology, with joint appointments in the Taussig Cancer Center and the Departments of Neurosurgery and Pediatrics at the Cleveland Clinic.

Agenda Item: Metabolic and Other Genetic Syndromes

DR. COHEN: Thank you.

Conflict of interest: I'm on the Scientific Advisory Board and Speakers Bureau for Transgenomic, which is a company that sells laboratory testing. The Department of Justice and my employer are under some negotiation about me working with the Vaccine Compensation Program.

I'm going to talk today about mitochondrial disorders. Really, the first half of the talk is going to be a more general overview of mitochondrial disorders, and

the second half is topics aimed at the purpose of this meeting today.

I want to start with mitochondria. These are the energy-producing parts of the cell. The classic view is that they are cigar-shaped objects about 1 micron in length, as shown in the upper right-hand picture of the cutaway cell. But in reality they are worm-like structures that are undergoing constant fission and fusion in real time. They really look like worms on a plate that are separating, budding, and then coming together again. So it's a very complex structure *in vivo*.

There are 350 different cell types in the human body and 350 different types of mitochondria.

The mitochondria are composed of an outer membrane and a heavily folded inner mitochondrial. An important concept is that there are about 1,500 different structural and enzymatic proteins that make up the mitochondria. The vast majority of these are encoded by nuclear DNA, which, of course, is inherited from each parent and undergoes recombination. That's the essence of Mendelian genetics. We inherit these after stages of recombination.

These proteins encoded by the nuclear DNA make up the codes for the proteins of fatty acid oxidation, urea

acid cycle, citric acid cycle, and the other matrix enzymes, several hundred matrix enzymes. The nuclear DNA encodes for the proteins that make up the inner and outer mitochondrial membrane structures, and the vast majority of the electron transport chain subunits are made up by nuclear DNA genes, of course, which must be imported into the mitochondria using chaperone proteins and then assembled with the other subunits, using a variety of nuclear encoded assembly proteins.

There are 13 proteins of the electron transport chain that are encoded by mitochondrial DNA. In every mitochondria, there are two to 10 copies of mitochondrial DNA. These are inherited *en bloc* without recombination from our mother. The ovum contains about 100,000 mitochondria, about a half a million copies of mitochondrial DNA. The spermatozoa contains about four mitochondria wrapped around its tail. If any of the paternal mitochondria or mitochondrial DNA ever makes its way into the oocyte at the time of conception, it is either overwhelmed by sheer numbers, copy numbers, or it is somehow deactivated. We are our mother's mitochondrial DNA, although, of course, we are not our mother's mitochondria, because the vast majority of the mitochondria again is inherited through the recombination events and

nuclear DNA.

Within this 16,569-base-pair circular protein, which is essentially all exon, there are 37 genes. There are two ribosomal RNAs that actually make 12S and 16S proteins that are remarkably similar to bacterial structures -- of course, the mitochondria are anthropologic bacteria -- 22 copies of transfer RNAs that again are a completely different genetic code than what goes on in the nucleus of the cell; and 13 structural proteins of the electron transport chain.

Two very important concepts are heteroplasmy and haplogroup. The heteroplasmy is the concept that when mutations in the mitochondrial DNA, they are not an all-or-none event. It's a graded percentage. One could inherit 93 percent of mutant gene along with the 7 percent wild-type or vice versa or any percentage in between. The degree of severity depends on the specific mutation, as well as the percent of mutant heteroplasmy.

The other concept is the concept of haplogroup. In the bottom right, you can look at L0, which is where humans began in sub-Saharan Africa. Over time, mutations have occurred -- these are viewed as non-pathogenic mutations, although we will find out that they may not be so non-pathogenic -- mutations have occurred that define

the haplogroup, as humans have migrated up into Africa and Asia, across the Bering Straits, so forth and so on. I am actually J2a haplogroup. My haplogroup has been tested as part of just my own personal curiosity. But we all are defined in terms of our mitochondrial heritage by haplogroup. We will get into how that may be important at the end or the talk.

This is a cutaway view of the electron transport chain. What isn't shown, which will be shown above, is the outer mitochondrial membrane. What sits between the outer and inner mitochondrial membrane is this potential space called the inner membrane space. On the lower portion, below what we see here, would be the matrix, the vast majority of the volume of the mitochondria. Embedded within the electron transport chain are the five enzymatic complexes, complexes I, II, III, IV, and V. You can see on the far left is complex I. It's quite complex. It's made up of about 50 different subunits, 41 or more of which are encoded by nuclear DNA, seven encoded by mitochondrial DNA. Buried within it are iron-sulfur clusters which help transport the electrons. There are iron-sulfur clusters in complex III. There's a copper cluster in complex IV. This is just to give you a general view of what the electron transport chain looks like.

UNEDITED VERBATIM TRANSCRIPT

I would love to talk to you about the biochemistry, but that's not what we are here about.

What do mitochondria do?

- Number one, they generate ATP, cellular energy.
- They are a critical component of apoptosis and, in fact, may be central to the process of how the body deals with viral infections, as the key importance of the mitochondria.

- They generate free radicals that are important for both health and disease.

- They serve as a genetic barometer that allows for both intermediate intracellular changes that occur over seconds to minutes to days, which would include the process of apoptosis, and quick evolutionary changes, measured over the course of thousands of years as opposed to hundreds of thousands of years. Mitochondrial DNA mutates much faster than nuclear DNA mutates. This mutation has probably allowed humans to have migrated out of sub-Saharan Africa. Humans can survive in very extreme heat and extreme cold. This may be an effect of haplogroup.

- The mitochondria serve roles in most neurodegenerative diseases and some cancers.

What are mitochondrial diseases? This I took from a paper that we wrote about two years ago. On the far

left are the red-flag findings of mitochondrial diseases. In Table 2 is nonspecific findings. In this table you can look at the neurologic, cardiovascular, ophthalmologic, GI, and other characteristics of mitochondrial diseases.

I'm going to simplify this a bit and show you a slide of mitochondrial failure.

Again, if you just look at the brain alone, everything listed here is basically the reason we need neurologists and psychiatrists in the first place. Pretty much anything that can go wrong with the brain does go wrong or potentially goes wrong in mitochondrial diseases. The muscles can be affected. The liver is a target organ. The peripheral nerve is another target organ; GI, GU, eyes, ears, systemic, and endocrine.

Essentially, these are the cells of the body that require a lot of energy, as well as the cells of the body that are postmitotic at the time of birth. These are not the cells that undergo constant replication.

I'm going to talk now about a few classic mitochondrial diseases. There are well over 100 named mitochondrial disorders, but I'm going to talk about the ones that are embedded in the history of mitochondrial diseases and the ones that tend to be more common of the named mitochondrial disorders.

The first is Leigh syndrome, first described in 1951. Of course, it wasn't attributed to a mitochondrial disease back then, because there was no such thing as a mitochondrial disease. Typically these children are perfectly healthy until -- in this child, the age of three, but it could be six months and it could be six years, and we have actually seen adults who have their first Leigh syndrome attack as adults. This young girl was well into the age of three. I looked at videotapes and pictures of her. I can attest by my inspection of these that this was a perfectly normal child. She had a nonspecific viral infection and at the tail end of the viral infection, she lost the ability to walk because of ataxia. She was admitted to the hospital. This MRI scan was performed. You can see that the midbrain was totally wiped out by this disease process. After the MRI was performed, the pediatric neurologist ordered a lactic acid measurement, and that was elevated. Really, within a few hours of her clinical presentation, she was properly diagnosed with Leigh syndrome. It's not a hard diagnosis to make.

During the hospitalization, she developed a hemiparesis and then another hemiparesis on the other side and was left quite devastated. Over the course of that hospitalization, she developed an eye-movement disorder and

bulbar dysfunction, lost the ability to swallow, and then actually made some recovery. During the recovery phase, she developed dystonia and neuropathy and has undergone deterioration since that point.

Leigh syndrome is a common final pathway to a number of different mutations, both mutations in the mitochondrial DNA and in the nuclear DNA. This has been a frustrating case, not only because of the deterioration, but because of the fact that I have done every test I can possibly think of, looking for the mutation in this disorder and haven't been able to find it.

Interestingly, the muscle biopsy is most often normal or non-diagnostic, except in those children with the complex IV defects. I tend not to do muscle biopsies on patients with Leigh syndrome just because of the low yield.

If you have never seen a patient with Kearns-Sayre syndrome, this is a young man with Kearns-Sayre syndrome. This is an incredibly easy diagnosis to make, because not only is there ophthalmoplegia, but there is heart block and high-frequency hearing loss. This takes about 10 seconds to diagnose in the office, the second time around.

Other features would include myopathy, diabetes, retinopathy, dementia and seizures, cardiomyopathy,

dysphagia and weight loss.

If you take a look at the upper right, this is just a cartoon of the circular mitochondrial DNA. Just keep your eye on that picture. In Kearns-Sayre syndrome, there is a 4,977-base-pair loss. This is a sporadic event that occurs at some point probably in the maternal oocyte when the mother before four months of age. We don't really know why that happens. But these are sporadic events.

Kearns and Sayre described this finding as an ophthalmologic disorder in 1958. It was better characterized by Bob Daroff in the 1970s as a neuro-ophthalmologic disorder. This is another progressive disorder.

MELAS is a disease that was depicted in the film *A Few Good Men*. That's what the marine recruit died of. MELAS has a variable age of onset and is usually due to one of two point mutations in the mitochondrial transfer RNA, leucine RNA. When it presents in infancy, it presents as failure to thrive and developmental delay. But most patients don't present in infancy. When it presents in young childhood, attention deficit disorder is a common feature. When it presents in the first few years of life, the children often present with Wolff-Parkinson-White syndrome. But again, this can present at any age. In

adulthood, it presents with weakness, fatigability, short stature, stroke and stroke-like episodes, which are a hallmark feature of this disorder.

I should say that MELAS stands for mitochondrial encephalopathy, lactic acidosis, and stroke-like syndrome.

These folks often undergo a progressive dementia. Hearing loss and diabetes are part of the illness. Interestingly, there are subsets or families of patients with this mutation that only have hearing loss and diabetes, and don't have anything else.

These folks can develop severe anorexia from autonomic gut neuropathy, migraine, and seizure disorder. Renal tubular acidosis is another feature.

In a Finnish study, the gene frequency for this disorder was 1 in about 6,000. Again, there are two common mutations that cause MELAS.

MERRF is the mitochondrial encephalopathy with ragged red fiber syndrome. When it presents in youth, it presents often with myoclonic epilepsy. But I have a patient I diagnosed at age 65. She had a myopathy, ataxia, and fatty deposits on her neck. It's a highly variable disorder. This is a mutation of transfer RNA of lysine, not leucine.

Leber hereditary optic neuropathy is generally

due to mutations in mitochondrial DNA complex I genes. Unlike everything else we have talked about in terms of being heteroplasmic, in most of the cases these are homoplasmic mutations. In other words, there is a 100 percent mutant load. This is felt to be due to the fact that these disorders aren't going to present in a heteroplasmic state because they are just milder disorders.

Leber is a painless, profound visual loss presenting in the late teens or 20s. There is a recovery in about 25 percent of patients. Interestingly, for those patients who present who are smokers and drink alcohol, if they give up the smoking and drinking, they have a higher rate of recovery.

Aside from some of these folks developing heart blocks later on in life, the extent of the disease is generally the visual loss, and pretty much nothing else.

How common are mitochondrial disorders? Pat Chinnery did an experiment. He actually looked at the 10 most common mitochondrial DNA mutations. Those are listed on the left. The 1555 mutation is the aminoglycoside-induced deafness mutation. He looked at the common MELAS mutations, several Leber mutations, and some Leigh and MERRF mutations. He just chose 10 mutations. He looked at 3,000 consecutive births in a northern England hospital and

looked for mutations.

What he found was that about 1 in 200 live births carry one of these 10,000 mitochondrial DNA mutations, although the degree of heteroplasmy is low in many of these folks. He also looked at the mothers and found that many of these were *de novo*. In other words, they weren't present in the mother. The *de novo* mutation rate for these 10 most common mutations is slightly over 1 in 1,000.

These are actually very scary numbers, because that means 1 in 200 people are carrying around one of these 10 pathologic mutations.

The most severe mutations are the MELAS mutations and those labeled in black down at the bottom. Those are the three black dots at the bottom. You could say, what's the chance of any of those people developing MELAS with a 2 or 3 percent heteroplasmy? Again, if you are 2 to 3 percent heteroplasmic, you are not going to develop MELAS, but if you are a female, within that female's ovum are already eggs that are waiting to be fertilized that have much higher mutant loads. The way heteroplasmy work is that there's a bell-shaped distribution of the degree of heteroplasmy. This is where we think the *de novo* MELAS patients come from.

Moving on to Alpers syndrome, in 1931, Bernard

Alpers, who is pictured on the left, described this fatal infantile disorder that he labeled as poliodystrophy. These children presented with seizures, horrible seizures, developmental regression, cortical blindness. The usual age of presentation was between three and seven years. Some of these kids were intellectually normal at the time of discovery; others had developmental disabilities.

In 1975, there was a single study showing mitochondrial electromicrographic changes in Alpers patients, and in 1976, Peter Huttenlocher described hepatic features in these patients. This became known as the Huttenlocher variant of Alpers syndrome.

Based on the pattern of illness, Huttenlocher described this as an autosomal recessive disorder. He also underscored the fact that many of these children presented with *epilepsia partialis continua* or *status epilepticus*. These children developed a progressive neuropathy and spasticity, and death one to 20 years within the course, often from liver failure. What we know now is that some, if not all, of the valproate-induced liver failure is in patients with Alpers disease.

The gene for Alpers disease was cloned and characterized in 1996 by Bill Copeland at the NIH. The disease was linked to Alpers in 2004 by Robert Naviaux,

whom I failed to thank in the first slide. He helped me develop many of the thoughts that I have regarding mitochondrial disease and vaccines.

Just jumping ahead four or five years, from the discovery of the first mutations in POLG. I failed to mention that this gene is polymerase gamma. This is the polymerase from mitochondrial DNA. Mitochondrial DNA undergoes replication constantly in our bodies, and POLG is the gene encoding for pol gamma, the only polymerase for mitochondrial DNA.

From the first discovery of the first mutations, which are the ophthalmoplegic mutations -- that was in 2001; those are listed in red above, all the other mutations listed below. Again, everything above started in 2001. Everything below the gene line began in 2004. Here are over 150 mutations known to be associated with these POLG-related diseases. I'll show you a slide of what POLG-related diseases look like now. It's not just Alpers disease or ophthalmoplegia.

Patients with POLG disease present with psychiatric symptoms, seizures of every type, extrapyramidal movement disorders of every type, including Parkinsonism and chorea, cerebellar ataxia, the cerebrovascular -- these are the migraine and stroke-like

episodes. They can present with demyelinating neuropathies, axonal neuropathies, muscle disorders, peripheral nerve disorders, endocrine disorders, GI disorders, and cardiomyopathy.

This is a gene. These mutations are fairly common. I'll show you that in the next couple of slides. This is a very important gene in terms of mitochondrial disorder.

How common are these disorders? I told you that the gene frequency of MELAS in the human population is 1 in 1,000, but, in fact, that most of these have such low degrees of heteroplasmy, they never cause clinical illness. A conservative estimate, a rational estimate, of the disease frequency of MELAS due to the A3243G mutation is about 1 in 5,000 humans.

If you look at POLG and you look at the Western European population -- and that's the only population that has really been extensively studied -- the allelic frequency of a pathogenic mutation in POLG is about 2 percent. Two percent times 2 percent times one out of four, since these are autosomal recessive disorders, gives a disease frequency of about 1 in 10,000.

If you add these two numbers together -- I'm going to tell you that the estimated disease frequency of

only the two most common mutations that cause mitochondrial disease is about 1 in 4,400. I haven't even told you about the other 100 named diseases yet. So these are not uncommon disorders.

However, when you go to the clinic, the majority of those that have been diagnosed with a mitochondrial disease do not have Leigh syndrome, Kearns-Sayre syndrome, MELAS, MERRF, Leber's, or Alpers syndrome. When you go to the mitochondrial conferences with the patients, the vast majority do not have any of these named low-hanging-fruit disorders. In most centers the ability to make a firm genetic diagnosis is in the order of 10 to 15 percent, and in the clinic we must live with the diagnostic uncertainty about what is and what is not a metabolic disease, since we are not able to get a genetic diagnosis in everyone.

We can't a genetic diagnosis in everyone for two reasons. The first reason is that even if you spent all the money in the world, there are only about 47 genes that you can test for. The second reason is, we don't have all the money in the world. Depending on the medical center you are at or insurance coverage that the patient has, testing for these disorders may or may not be done.

So how do we reach a diagnosis?

Number one, we take a clinical history and we

look for the red-flag signs of mitochondrial disease. We look for a family history, especially for maternal inheritance. That is quite important in the mitochondrial DNA disorders.

We do a physical examination, looking for neuropathy, myopathy -- all the things that I have talked about.

The inexpensive tests -- and I say inexpensive not because they are pennies, but because they are not tens of thousands of dollars -- are the analyte tests. We are looking at blood, urine, and spinal fluid for biochemical markers of mitochondrial dysfunction. We can actually look at the end organ. We can look at the MRI scan, which sometimes has classic features of mitochondrial injury. We can do echocardiograms looking for heart failure, for example.

Those are rather standard tests that are neither necessarily specific nor sensitive.

Then, when we get to the big-buck testing -- and these are the invasive tests -- we can do tissue histology. We do muscle and skin biopsies looking at microscopy, immunohistochemistry, protein studies. We can actually do biochemical studies on the mitochondria, looking at integrated mitochondrial function using polarography, or

enzymatic function using spectrophotometric methods, and then the molecular genetics.

Just to give you an example, I build my practice on doing rather intensive evaluations of patients. This is my typical lab evaluation that I do on a patient that I really feel has a mitochondrial disorder. I would order these blood tests. I would order these urine tests. Pretty much all the patients get that type of testing done. I would ask myself the question, is the child dysmorphic or have cognitive problems? If the answer to that is yes, we are doing array CGH microarrays. That has about a 15 percent final diagnosis detection rate. That's a very important test, the cost of which has come down considerably in the last five years.

I also ask myself the question, could this child have any of the well-known, described neurologic conditions that can mimic mitochondrial disease, like fragile X, Rett syndrome, Prader-Willi, Angelman, the CSF neurotransmitter disorders? If the answer to that question is yes, I may just do specific genetic testing for those disorders.

Then I ask myself the question, does the patient look like they have a specific mitochondrial disorder like MELAS? If the answer to that question is yes, I'll order the specific mitochondrial DNA point mutation. If I think

the patient has Kearns-Sayre syndrome, I'll do a Southern blot looking for the large-scale deletion.

If it looks like it's a nonspecific mitochondrial spectrum disorder with a maternal inheritance pattern, we can now do a whole-genome study and haplogroup typing on the mitochondrial DNA. Again, this is a test where the cost has come down considerably in the last five years. It used to cost about \$8,000 and take about a year to get the answer back. Now we can get the answer back in a week or so, at about one-third the cost of doing an MRI scan.

If the child or adult looks like they have a specific disorder of nuclear DNA -- again, I think there are about 47 different genes that now we can order testing on, and 47 different syndromes -- then I can actually do gene testing on that.

So you can see that we are talking about a lot of testing, at a considerable expense. And this doesn't even include looking for end-organ dysfunction, like brain and heart. Sometimes I'll do a skin biopsy as a less expensive, less invasive set of testing.

We have to look for other things besides mitochondrial disease, because again there are mimickers -- the neurotransmitter disorders, the disorders of peroxisomal metabolism, disorders of lysosomal metabolism.

So this is not an easy diagnosis to make.

In the end, when we go to the mitochondrial meeting and take a look at all the patients that have been diagnosed with mitochondrial diseases, we find a lot of false positives:

- One reason is failure to recognize well-characterized genetic disorders that aren't mitochondrial. Some of these are common, like DiGeorge syndrome, some of these are rare, and some of these are extraordinarily rare.

- Improper sampling handling, whether it be blood giving you a false-positive lactic acid elevation or muscle. If you don't handle muscle properly and do enzymatic analysis, the enzyme function disappears in 20 minutes, so you get enzyme levels of zero and the patient is improperly labeled as having a mitochondrial disease.

- Poor laboratory technique.

- Incorrect interpretation of lab results. I see this all the time. A little bit of knowledge can be dangerous. Labs report mutations of uncertain significance or of no significance, and these people are labeled incorrectly as having a mitochondrial disease based on these results.

- Lack of doing proper testing. You rely on a clinical history alone.

- Children and adults who have disuse of muscle will have false-positive decreases in mitochondria enzyme function. If you take a healthy person, with normal electron transport chain enzymology, and you put them in a leg cast and you biopsy their muscle two weeks later, there will be a 25 percent reduction in enzyme function. The same thing would be true of a child with hypotonia who doesn't use his leg muscle or a child with cerebral palsy, for example. So relying on enzymatic function of electron transport chain activity has been -- we are now recognizing this as a real problem in relying on this as a diagnostic test alone.

I could go on and on and on about the false positives, but I'll just move on.

We have some unproven suspicions. These may be also referred to as difficult truths to accept.

The common final pathway to all cellular demise may be a mitochondria-triggered event. The person who is best known for his work in pyruvate dehydrogenase deficiency, Doug Kerr, jokes that everyone dies with lactic acidosis. It's true.

Common disorders may have at the core of pathophysiology mitochondrial dysfunction. The corollary of this is that everything is a mitochondrial disease.

Again, that may be true to some extent, but it doesn't get to the root of the problem.

Finally, a fascinating point being pushed by Bob Naviaux is that the innate immune response may be a key mitochondrial function, and so a reaction to any infection may, in fact, create a cascade of mitochondrial dysfunction.

I was asked these four questions:

- Natural history of the disorders.
- What percentage of children with mitochondrial disease present with encephalopathy?
 - Is the long-term prognosis for children with these disorders who have an acute encephalopathy different or worse than children without the acute encephalopathy?
 - Are there developmental time periods at which the child or adult may be more susceptible?

The animal cell mitochondrial proteome is tissue-specific. There are 350 different cells in the body and there are 350 different types of mitochondria, each doing something different. The mitochondria in our brain, which have to support the sodium-potassium-ATPase pump -- that job is to produce as much ATP as possible without a break, 24/7. That's different than the mitochondria of our cheek cell. It doesn't take much ATP to be a cheek cell. What

that cheek cell needs to do is replicate itself as fast as possible. So the reducing equivalents in a cheek cell aren't shuttled down into the electron transport chain. They are shuttled into making, not NADH, but NADPH, the ribose-phosphate shunt to make nucleotides so that they can double their cell mass and divide and divide and divide. Again, very different things that mitochondria do in different cells.

But we are really here to talk mainly about the brain. Why the brain is so susceptible probably has to do with numerous factors, including the fact that it does not have a store of ATP and can't survive without ATP being made on a heartbeat-to-heartbeat basis.

One of the new concepts that we are looking at in mitochondrial disorders is mitochondrial DNA copy number pre cell. This tends to be regulated by developmental state and cellular needs. Up until now, there was no easy way to measure it, but there will be new ways coming online very quickly to measure mitochondrial DNA copy number. We are looking forward to this, because we could do this in real time. We are talking about doing this at a baseline state, at a day into a runny nose, five days into a runny nose, and a month after a runny nose.

Mitochondrial DNA content may be a biomarker of a

number of other disorders. Again, when you have a test that becomes cheap and easy, you can really apply it in a variety of different ways.

One reason this is not going to be such an easy thing to figure out with mitochondrial disease and vaccination is that we're not talking about one disease; we're talking about hundreds of different diseases. If you take a look at a single respiratory chain subunit of complex IV, called COX4, in a yeast, if you knock that out -- again, there were 2,376 different proteins, 40 percent of which changed more than 150 percent with decreases and increases in protein. Again, if you go knockout by knockout, you are getting different effects. This cascade effect is really well described in these biochemical pathways. Again, if a thunderstorm appears over Newark Airport and lasts 30 minutes, within an hour air traffic in San Francisco has changed.

I'm going to get back to the mitochondrial DNA haplogroups. Up until recently, these were thought to be benign mutations that just defined where someone's maternal heritage came from. But we are now seeing that these confer risk and resistance to disease. The mitochondrial DNA structure itself is inherited *en bloc* via our mothers. These contain the 13 different electron transport chain

enzymes, which are the core enzymes of the electron transport chain. These are inherited without recombination.

Nuanced differences in base-pair substitutions probably confer a balance between coupling of oxidation and phosphorylation and uncoupling, to help suit the environment and help adapt to temperature elevation, sunlight, water availability, food resources, and infectious agents. This is a concept being pushed forward by Doug Wallace at UCI. These root mitochondria DNA changes may represent minor but fundamental tradeoff decisions in fitness. There are dozens of examples that exist.

One of the largest studies done was a study out of Vanderbilt, published by Cantor in 2007, where he looked at 745 consecutive patients admitted to the ICU for trauma. They had mitochondria DNA on 666 of these patients. There was a mutation in a complex I gene called ND1. Again, it's a mitochondrial-inherited gene. The mutation can be associated with Leber's hereditary optic neuropathy. In the majority of patients who did not have the mutation, the wild-type -- that's the T4216 -- there was an increased mortality from the trauma, with an odds ratio of 2.63. These folks also had increased male fertility and decreased

diabetes type 2. The ones with the mutation -- that was found on haplogroups J and T -- had decreased traumatic mortality. These folks also had decreased male fertility and increased insulin resistance and DM2. So if you can make it off the battlefield, you are more likely to reproduce. That's another way of looking at it.

This same mutation was studied in another study, published very recently, another trauma study, looking at the odds ratio of 3.68. So this has now been looked at in two different studies.

There was a *Lancet* article in 2005 which looked at infection. Pat Chinnery took 150 sequential patients admitted for sepsis. He had 542 age-matched controls. He was bale to do mitochondrial haplotyping. The endpoint was death versus survival at six months. In the 150 and 542 different groups, there was no difference in the frequency of haplogroup H. Haplogroup H is the most common haplotype in northern Europe. Those belonging to haplogroup H -- it was a predictor of improved survival, conferring a twofold increase in survival at 180 days.

Interestingly, haplogroup H is the most recent addition to the haplogroups in Europe, but, paradoxically, the most common.

These are several studies, and there are many

more, showing very different risks to environmental events in terms of survival or not.

Getting back to the natural history, I'm going to go into this in a little bit more detail. If we look at encephalopathy at presentation -- in other words, the first event being an encephalopathic event -- and cognitive delay prior to onset, in the patients with Leigh syndrome, these are normal children prior to the onset of the disease. They present with encephalopathy.

In MELAS, some of these kids, before they actually present, have attention deficit disorder or mild cognitive delays, with encephalopathy being a quite common presentation.

In Leber hereditary optic neuropathy, there is no encephalopathy and there is no cognitive delay. These are cognitively normal people that remain cognitively normal.

Kearns-Sayre syndrome really presents with encephalopathy. It presents with ophthalmoplegia, high-frequency hearing loss, and cardiac conduction defect. But there is a subgroup of patients with Kearns-Sayre syndrome that have cognitive delays. Pearson syndrome is the infantile presentation of Kearns-Sayre syndrome, and all these children are cognitively delayed.

Alpers syndrome, again due to polymerase gamma

mutations, they present with encephalopathy. That's the most common presentation. But many of them have what were viewed as static cognitive delays prior to the presentation. They were slow in school. They were slow in terms of their language development.

If you look at the microdeletion disorders -- these are the disorders where you have a 1-megabase to 5-megabase deletion in a nuclear DNA segment -- the vast majority of these kids have cognitive delay. Of course, the reason we order this test is because of dysmorphism and cognitive delay. I can't think of a single patient that presented with an encephalopathic event, although that's certainly possible.

The mitochondrial DNA disorders are progressive. They aren't all progressive in terms of encephalopathy, but they are progressive disorders. Again, Kearns-Sayre syndrome, depending on how severe it is, is a fatal disorder, as is MELAS, as is MERRF.

Having said that, I told you I had this patient aged 65 who presented with MERRF.

Leber is a progressive disorder, but the progression takes place over the course of months. Once the vision is lost, that's it. There doesn't seem to be any progression after that, except for the patients that

will develop an occasional cardiac induction defect. There are some, actually, that develop some dystonia.

The severe nuclear DNA disorders are typically progressive. There are lots of them.

I didn't mention the disorders of fatty acid oxidation, the disorders of citric acid cycle and the urea acid cycle enzymes. These are disorders that are easily diagnosed on almost every state's newborn screening program. What we know about them is that if you have a catabolic event, it can be fatal. So these are quickly fatal with stress. I'm going to take that out of the picture, because those are screened for at two days of life.

Again, the disorders defined by array CGH microarray technology -- you can knock out mitochondrial proteins in a deletion. This is a topic that the mitochondrial doctors are all on top of, but haven't gotten all on top of it until a year or two ago. For the most part, these tend to be non-progressive diseases. If you have a microdeletion, it is what it is, and it's not going to get any worse.

I like to divide the brain disorders into embryopathies and acquired. You can have mitochondrial disorders that are embryopathic at birth or prior to birth,

but we think most of these are the microarray disorders. These present with global developmental delays, atypical CP, autism or pervasive development disorders, and seizures due to disorders of neuronal migration. The children with mitochondrial disease that have acquired brain dysfunction -- and that's the encephalopathy -- are generally normal at birth through presentation, but can present with seizures, atypical migraines, stroke and stroke-like episodes, neuropsychiatric symptoms, and dementia.

This is a patient -- sometimes it isn't so easy to divide the two. I have been following her since 1992. I met her when she was four. She presented on the backdrop of a static cognitive delay. You would look at her now and you would say this child is autistic. But at that time we called it a static cognitive delay. She presented with seizures and hemiplegic migraine events. We found lactic acidosis. This is what her MRI looked like. It shows a colpocephaly. This is a sort of neuronal migration. You can see some pachygyria as well.

We did an evaluation, and we believed her to have an electron transport chain complex I defect. She remained stable for a period of time, until she had a profound encephalopathic event. Mother said that it started on a

Sunday. She woke up. She didn't feel quite right. The girl wasn't acting quite right. They went to a church picnic. It was 95 degrees outside. They laid her down under a shade tree, and she slept for several hours under the shade tree -- again, 95 degrees. When they went to wake her, they couldn't wake her up. She was in a coma. She remained in a deep stupor for about a month. Her MRI showed severe demyelination. She recovered in a month and she got back to her baseline.

I referred her to the University of California, San Diego, for their dichloroacetate trial. She has been on dichloroacetate since the late 1990s, and she has been stable. There is no dementia.

I have done an extensive genetic evaluation on her, trying to figure out what's going on, and haven't gotten anywhere with that genetic evaluation.

So here's a girl who has signs of both an embryopathic brain and acquired but reversible encephalopathy, heat stress-induced.

What can we say about the rate of progression? Even for a well-characterized disease like MELAS, MERRF, Kearns-Sayre, or POLG, there is a huge and unpredictable variation in progression.

I follow an extended family with MELAS. The

sickest child died before the age of 10, with essentially such a bad renal tubular acidosis that the ICU couldn't keep the sodium above 120 or the bicarb above 14 with sodium bicarbonate infusions, including a 3 percent saline infusion. There are other family members that have equal degrees of pathogenetic heteroplasmy that are perfectly healthy until their adult years.

One of these patients and her fiancé went on a trip to Las Vegas in August. I said, "Whatever you do, stay cool and stay hydrated." The last day, they had to check out of their hotel at noon. They spent the day on the Strip, where she became dehydrated. She started vomiting before she got on the plane ride back to Cleveland. Her fiancé brought her to the hospital when they arrived at about 11:00 at night, again vomiting. She died in bed that night, probably of an electrolyte disturbance, but it was unclear.

So again, to just make the point that there are stressors that can kick patients over from being able to walk up and down the Las Vegas strip in August to being dead within 24 hours.

There is a huge spectrum of survival in Kearns-Sayre syndrome that we don't understand. Similar to POLG, I have patients with identical mutations, and one guy is 43

years old and is up and about complaining of fatigue -- he has problems, but he is able to get around and hold down a job -- and other patients with the same mutations that die within weeks after presentation.

What percent of children with these disorders present with encephalopathy? Almost by definition, Leigh syndrome is encephalopathic in its presentation. MELAS approaches 100 percent. In Kearns-Sayre, it's rare as a presenting sign. In Alpers, it's about 100 percent. In Leber, it's about zero.

Again, the children with the microdeletions found on array CGH testing have static cognitive delay -- I would say over 90 percent.

Here's where the question actually is longer than the answer and a lot longer than the data to support the answer: Is the long-term prognosis for children with these disorders who present with or experience an acute encephalopathic event different or worse than children without the disorders that present with or experience an acute encephalopathy?

Point one: Children with embryopathic brains tend not to have dementia, unless it's an epileptic-induced dementia. These tend to be the kids with the static course and seem to be less susceptible to the metabolic stressors

and tend to be the ones where, if you find a mutation, it's going to be the microdeletion type. Children with normal brains that have acute encephalopathy and are found to have mitochondrial and nuclear DNA mutations that cause their mitochondrial disorders tend to have a worse overall prognosis than children that have had an extensive evaluation and we can't find the mutation.

Again, I think this is partly because the sickest patients have reflexively allowed laboratories to do more extensive searches for their mutations. You find the more serious mutations, the more common mutations earlier, before you find the less serious and less common mutations.

What about the developmental time periods? Again, those with the fatty acid oxidation, citric acid cycle, urea acid cycle, organic acidemias tend to present earlier on, with more catastrophic events. But these are also the disorders that are screened by the newborn screening programs. The mitochondrial disorders have such a wide degree of disease presentation, it makes it difficult to make a statement. The more severe the disorder, the earlier it presents. But if you remove this age factor from the variable, age doesn't seem to be a variable. Again, with the nuclear DNA disorders, there is such a wide variable age of disease presentation that it's

difficult to make a statement.

Here are the stressors that we worry about as clinicians:

- Infection.
- Temperature. By temperature, I mean fever, I mean ambient room temperature, humidity issues.
- Dehydration stress is critical.
- Starvation, catabolic stress is critical.
- We find that sleep is a very important factor.

A lot of our patients have sleep apnea, and when we correct that, either through surgical manipulation of the airway or using BiPAP machines, the patients function better, are less encephalopathic.

- There are many mediations that cause mitochondrial failure. Some of these have been mentioned today in the talks -- the volatile anesthetics. Propofol is a potent inhibitor of mitochondrial function. Some of the antibiotics are potent inhibitors of mitochondrial function, and certainly some of the cancer chemotherapy agents.

In terms of a practical approach that we take in our patients with vaccinations, I would say, with no outliers in terms of clinicians, we do our best to get our patients fully vaccinated, using the standard vaccination

schedule. With our patients whose parents are hesitant, we will talk to the pediatrician and say, okay, to get this in, let's break the vaccines up, not giving four at once, but doing it one at a time -- although I tell the parents and tell the pediatrician, as far as I'm concerned, if you take the global physiologic stress, it's probably less physiologic stress to give four shots all on one day than to give one shot once a month.

Sometimes we will add ibuprofen just to suppress the immediate reaction, although, again, I tell the parents and the pediatrician that we are not going to give ibuprofen for a month, and the immunity process that goes on -- pardon me, I'm not a virologist or immunologist, and I may not be using the right terms -- that's something that is going on for many, many weeks after the injection.

So sometimes we do these manipulations in order to get the kids vaccinated. But in my heart of hearts, I don't think that makes any difference or is any safer than doing it in the standard fashion.

Our knowledge is only the tip of the iceberg. I hope I have answered your questions the best that the literature supports. Thanks.

Agenda Item: Discussion

DR. CLAYTON: Before I open it up to the rest of

the committee, I would ask you to speculate about this. One of the things that we are wondering is, how can you tell whether a vaccine causes a child's encephalopathy or whatever to be worse than it otherwise would have been in the absence of vaccine? Given your approach to thinking about metabolic medicine, how might you approach that?

DR. COHEN: If we look at the slide up here with the most common identified disorders, I haven't seen any encephalopathy due to vaccines in any of these patients. I have seen one child who had a case heard in the Vaccine Court, who was diagnosed with a mitochondrial disease on the basis of an encephalopathic event that occurred within, I think, two weeks of a vaccination. I have seen that on one occasion. But I personally have not seen -- again, there's nothing worse than Leigh syndrome. That's the worst of the worst mitochondrial diseases. Every patient I have seen with Leigh syndrome has undergone full vaccination schedules, up until the point of their disease, without any events. The same would be true with my MELAS patients and my Alpers patients and my Kearns-Sayre patients and my microdeletion patients.

So I don't think there is any way to answer your question, because the events themselves are so rare.

DR. CLAYTON: Martha?

DR. CONSTANTINE-PATON: You mentioned haplotype H in the northern Europeans. Are there any other haplotypes that seem to be more susceptible to any of these diseases or to vaccines? I guess it's going to be the same answer that you just gave.

DR. COHEN: Where a lot of the study has been done is in Europe. It would be real interesting to go to sub-Saharan Africa and look at the haplogroups L.

One of the speakers, and I forgot who, mentioned varying the vaccines based on genetics. I don't know how you do that in the first place, because the only way to find genetics is to do extensive genetic testing. You can't use skin color anymore. Skin color has very little to do with anything.

DR. CONSTANTINE-PATON: But are the haplotypes easy to test for?

DR. COHEN: Oh, yes. Again, you can spit into a -- 23andMe, for example, will tell you your exact mitochondrial haplotype. They have to look at, I think, probably about 50 different points on the mitochondrial DNA to tell you what your haplotype is. I'm not trying to be a proponent for 23andMe. But for the cost of \$399, for which they are making a profit, they can give you that and 50 other pieces of information.

I can't tell you what it costs a laboratory to do haplotype testing, but my guess is that it's not that expensive.

DR. DIAMOND: I have two questions. One is, it seems to me from everything you have said that there is a pretty close temporal relationship between a stressor and an adverse outcome in these diseases. I want to know if that's true or what you think the longest time between a stressor and an observable outcome is.

Then the studies of sepsis were very intriguing to me. Most of what you described up until that point really were brain diseases. You made the point of the brain being the most susceptible organ. I don't know what the people who die with the sepsis are dying of. But I was wondering if you think that, in fact -- depending on the virus and if it's a live virus or something -- you could have a lung disease from mitochondrial defects because of lung infection, without any concomitant brain disease.

DR. COHEN: The first question has to do with the stress causing the first presentation. I think it would be fair to say, with Leigh syndrome, that the vast majority of identifiable stressors that lead to the encephalopathic event are simple viral illnesses. But when you look at the patients who present with Leigh syndrome, most don't have

any history of recent infection.

So number one is, there is no identifiable stressor. Number two is, the stressor is a common viral illness -- and I guess bacterial illness, but we are talking about non-critical bacterial illnesses -- strep throat, for example.

The temporal relationship is that it appears to be at the tail end of the viral illness. It's four, five days into the course. It's not at the maximum time of the fever. It's not when the kids are the most dehydrated. It seems to be a little bit later than that.

DR. DIAMOND: (Off-mic)

DR. COHEN: Let me think about that. If you take someone with early Alpers syndrome -- so before they have had the dementia -- they have their first seizure and you put them on Depakote, their brain will be functioning at 95 percent when their liver poops out, when they are in fatal liver failure.

So the answer is yes. When I think about the patients that have died, they have either died suddenly of unexplained -- when I say unexplained, I mean firmly unexplained causes, like the woman who went to Las Vegas and died in our hospital. We don't know exactly what the event was. Did she have a heart arrhythmia? Was it an

electrolyte disturbance? We don't know. It wasn't an electrolyte disturbance that you can measure on a CMP. We don't know.

But of those that you can identify, it's almost always brain. People will develop diabetes and exocrine pancreatic failure, but they don't die of diabetes or exocrine pancreatic failure. Cardiac disease is a common mode of death in Kearns-Sayre patients. They die of either a heart arrhythmia or cardiomyopathy, and not of the encephalopathy.

But I would say it's almost always encephalopathy.

DR. WEINER: I'm just wondering if people have looked at stress proteins in relationship to heat, particularly, or in terms of infection -- heat-shock proteins and that whole group of proteins. Those are very often associated with stresses.

DR. COHEN: Not that I'm aware of, in terms of the mitochondrial picture. The "mitochondriologists" really do have a hard time convincing our colleagues to jump on the bandwagon, because we are so far out in leftfield in terms of everyone's comfort level, in terms of both genetics and biochemical function that it's hard sometimes to communicate with our colleagues. I'm stating

the obvious.

DR. WEINER: The second question: I'm just wondering, are the mitochondrial genes susceptible to epigenetic phenomena -- methylation sites and histone deacetylase?

DR. COHEN: There are no histones in mitochondria.

DR. WEINER: That's what my question was.

DR. COHEN: They are much more prone to mutation damage because there are no protective histones. They sit in immediate proximity to the free radical generator, complex I. If a free radical is going to hit anything, it's going to hit mitochondrial DNA, before it even gets to the lipid layer. They are about 10 times more susceptible to mutation.

In fact, if you look at someone who has had a heart attack and look at the surviving tissue around the necrotic tissue, there are mitochondrial DNA mutations in the reperfused living areas of the heart. These mutations probably are the basis of the cardiomyopathy that develops after an MI.

DR. KOMAROFF: What's known about the mechanisms by which a viral infection can produce an acute mitochondrial dysfunction syndrome? I believe there are

some chronic viral infections, like enteroviral infections of muscle and cardiac muscle, that produce chronic, sub-lethal mitochondrial dysfunction. Do we know what the mechanisms are of either of those two?

DR. COHEN: There is a slide I don't have with me that I was trying to find last night, where I listed all the common viruses -- CMV, EBV -- and all of the targets that would be mitochondrial targets for them. Those are known. But in terms of a clinical correlate, that hasn't been put together at all. As far as I know, there's nothing that's known.

DR. CLAYTON: Please join me in thanking all three of our speakers. This has been very helpful.

Thank you to those of you who have listened in, both here and over the phone. As we said, we would appreciate your comments by email, if you are interested in sending them to us.

We will now close this session and go into closed session for the rest of the day. Thank you all again.

(Whereupon, at 1:15 p.m., the open session was concluded.)