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Conference

Meeting report
Part III. Notes from the 20th Annual Clinical Virology Symposium, April 25–28, 2004, Clearwater Beach, FL, USA

In the two previous issues, we described talks from the Molecular Virology Workshop, and the first two days of the 20th annual Clinical Virology Symposium (CVS, April 25–28, 2004), the latter attended by 727 clinical virologists and others interested in the field. Both meetings are organized by the Pan American Society for Clinical Virology (PASCV) and the University of South Florida College of Medicine, and have been put together for two decades by the same group of dedicated people. Like the Workshop, the CVS sessions are characterized by the high quality and focus of the presentations, lively question and answer sessions, and (not least) the same pleasant meeting location in a modest older hotel on a beautiful Gulf of Mexico beach. CVS is distinguished from the Workshop by the addition of posters and exhibits and by the panel discussion format of some of the presentations. A CD with the audio portion of all the lectures and discussions is available from IntelliQuest Media, Inc. (www.intelliquestmedia.com, Ref. #20-04).

In this final section of the report, we offer brief descriptions of the sessions from the last 2 days of the meeting, covering topics that ranged from updates on detection and treatment of several viruses, through case presentations and technology treatises, to vaccine development and disease eradication efforts. Learning opportunities were many and intense, and when the sessions were over, you could walk on the beach and ruminate on the day's lessons.

Michael Fried, MD (MF), **University of North Carolina**, Chapel Hill, NC gave the Fredrich Deinhardt Lectureship, speaking on Hepatitis C Virus: Evolving Guidelines and Paradigms. He cited CDC data indicating that 5% of men and 2.5% of women in the U.S. between 40 and 50 years old are infected, and then described recent therapeutic breakthroughs that result in a 50% cure rate for this otherwise largely chronic infection. Cure is defined as undetectable HCV RNA and normal ALT after 6 months of treatment, as only 1–2% of such patients relapse. Although the reasons for the difference are not understood, it is clear that HCV genotype is a major factor in response to therapy, and thus pre-treatment genotype determination is mandatory. About 70% of U.S. cases are genotype 1, which has a lower average response rate than other genotypes and requires more extensive treatment. MF's studies showed that a two-log reduction in viral load at week 12 of therapy predicted sustained response after 48 weeks of therapy in 65% of patients infected with HCV Genotype 1, whereas failure to achieve a two-log reduction predicted treatment failure in 97%. Thus their current treatment paradigm consists of initial genotype and viral load determination followed by re-test of HCV viral load after 12 weeks of treatment in Genotype 1 patients. If two-log reduction has not been achieved, treatment is stopped. If HCV RNA is undetectable, treatment is continued to 48 weeks without further testing. If HCV RNA is detectable, the test is repeated at 24 weeks of treatment: if at this point HCV RNA is still detectable, treatment is stopped. If HCV RNA is undetectable, treatment is continued to 48 weeks. Patients with Genotype 2 or 3 get 24 weeks of treatment without interim HCV RNA testing, and achieve a 78% sustained response rate. Minimizing treatment duration is important for limiting side effects and for efficient use of resources.

In the discussion, the following points were brought out:
The HCV core antigen test is not sufficiently sensitive to be a useful marker of early virologic response; its limit of detection is roughly equivalent to 60,000 copies/ml HCV RNA.
A third of HIV patients are co-infected with HCV. Their treatment side effects are more pronounced, and they have a 25–35% sustained response rate.
If you encounter mixed genotype infections, treat the most resistant genotype.
In sustained responders, HCV seems to be cleared even from liver tissues.
50% of children exposed via blood clear HCV spontaneously.

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Sara Miller, Ph.D. (SM), Duke University Medical Center Durham, NC reported on the detection of viruses in clinical specimens using electron microscopy (EM). First used in the late 1930s, EM is now extensively used in difficult viral diagnosis, and is the gold standard for the detection and identification of new pathogens. EM identified emerging disease agents such as SARS coronavirus, monkeypox virus, Nipah virus, Hendra virus and Ebola virus. In each case the pathogen was unknown and unsuspected and no prior information was required for analysis (as compared to molecular methods).

Molecular methods have become more sensitive than EM in the past decade. EM requires expensive instrumentation and trained personnel, but is still the fastest method to detect viral pathogens, and does not require cultivable agents. SM's lab tests approximately 1000 specimens/year for viruses, 90% in fluids and 10% in cells or solid tissue. Most of the specimens are diarrheal stools from gastroenteritis patients, since there are some enteric viruses that do not grow or grow very slowly in cell culture. EM testing provides an advantage in urine examination for polyomavirus, since most people were exposed to polyomavirus by age 9–10 and are positive for antibodies. SM frequently monitors urine (a non-invasive specimen) from kidney transplant patients, who are prone to contract polyomavirus or may have a reactivation. These viruses can contribute to organ rejection by activating the immune system, and thus increase the requirement for anti-rejection drugs. The most commonly seen polyomaviruses are BK and JC, which are not cultivable. Currently their detection is possible only by EM. SM found that these viruses are the causative agent for folliculocentric skin infection in immunocompromised hosts.

Negative staining of specimens (stool suspension, tears, urine), which is very rapid (10–60 min) and uses an electron dense heavy metal salt solution, is the most widely used EM technique. Liquid specimens are routinely concentrated by tabletop ultracentrifugation, which adds only 30–50 min to the preparation. EM is valuable in detecting adventitious viruses in tissue culture, and is particularly useful for examination of unknown and unsuspected agents in bioterrorism surveillance and the detection of emerging diseases.

Christine Ginocchio Ph.D. (CG) North Shore LIJ Center Health System Lab, Lake Success, NY talked about human papillomavirus (HPV) and provided a comprehensive update on HPV diagnostics. The human papillomaviruses belong to the family of papovaviridae and are the causative agents for different warts. HPV was first linked to human cancer in 1980 by Harald zur Hausen (Germany) and recognized by the WHO as a tumor virus and the major cause of cervical carcinoma and adenocarcinoma in 1996. HPV is the most common sexually transmitted agent in males and females, but is also transmitted from mother to child. Estimated incidence of new infections is between 1 and 5.5 million/year in the US. While most infections are spontaneously cleared, 5–10% of women older than 30 years are persistent carriers of HPV. Incidence and mortality has decreased in the U.S. in recent years, with approximately 4000 deaths/year currently due to cervical cancer, making it the third most common cause of cancer in women. Tumor progression is believed to occur in stages from first infection of the basal epithelial cells, to active infection, to sub-clinical latency, and finally advancement to tumor (from benign, to hyperplastic, to pre-malignant and malignant). Progression to cancer from HPV infection occurs due to blocking of the tumor suppressor proteins Rb and p53 through interaction with the HPV proteins E6/E7. The virus may either integrate into host DNA (mostly found in cancers) or is found as an episome (in sub-clinical latency). In 20% of cervical tumors the virus does not integrate. Two hundred HPV genotypes are known, and those associated with cervical intraepithelial neoplasia (CIN) have been divided into three categories: unspecified, low risk and high risk. The high risk genotypes with the greatest association with cervical carcinoma are 16 and 18. Of women positive for 16/18, 39% will develop CIN grade 2 or 3 within 2 years.

Cervical cancers have been traditionally diagnosed by cytology on Pap smears (Papanicoleau stained smears) according to the Bethesda Classification system and the ACOG (American College of Obstetricians and Gynecologists) recommendations. The sensitivity of detecting High grade Squamous Intraepithelial Lesion (HSIL) was shown to be enhanced by the addition of HPV DNA testing from 58% (cytology only) to 93% (cytology plus HPV DNA testing). Thus, the U.S. FDA (Food and Drug Administration) approved the combination of cervical cytology and HPV DNA testing for primary screening for women aged 30 years and older. Systems for HPV DNA detection include *In situ hybridization* (Benchmark and INFORM® HPV system, from Ventana Medical Systems, Tuscon AZ; DAKO GenPoint™ System, Trappes, France; InPath™ Molecular Diagnostics and Invirion, Frankfurt, Germany; the NASBA-based Pre-Tect HPV Proofer, Norchip, Norway); liquid hybridization (Digene Hybrid Capture® 2 HPV DNA Test), or by various PCR amplification and detection methods (Roche HPV Line Blot, Roche Diagnostics, RUO only and home brew applications, such as HPV ASR (analyte specific reagents)). CG also provided summaries of and references to several test methods and method evaluations: see Castle et al., 2004; Clavel et al., 2001; Gravitt et al., 2000; Gray and Walzer, 2004; Menezes et al., 2001; Pretet et al., 2004; Qureshi et al., 2003; Solomon et al., 2001; Wright et al., 2002; and ACOG Practice Bulletin vol. 45, August 2003). The goal of combined testing is to provide effective tools for the diagnosis and management of cervical dysplasia, to identify women at risk for progressive disease and detect cancers early, while reducing the number of unnecessary colposcopies.

Gregory Storch, MD, (GS) St. Louis Children's Hospital, St. Louis, MO discussed the history and diagnosis of human polyomavirus infections, beginning with the discovery in 1953 of mouse polyomavirus, and followed in 1960 by the identification of SV40 in rhesus monkey kidney cells used for the production of polio vaccines. The family of the polyomaviridae has 13 known members including 2 human viruses, JCV and BKV, both named by the initials of the

persons they were first isolated from. Polyomaviruses found worldwide have a narrow host range, can transform cell lines in culture and can cause tumors in some experimental animals. The small (5.1 kb) ds (double stranded) circular DNA encodes various proteins such the large T antigen, the small t antigen, the agnoprotein and the structural proteins VP1-3. The T antigen plays a crucial functional role through multiple enzymatic activities and interaction with host proteins (see also Hess and Brandner, 1997). Serological studies have shown that human infection with these viruses occurs during childhood, although the route of transmission is unknown. Seropositivity increases with age. JCV and BKV circulate independently. The virus may persist in various sites of the body including kidneys, brain and lymphocytes, and may reactivate with pregnancy and during immunosuppression. Human diseases such as PML (progressive multifocal leucoencephalopathy) are linked to JCV, and BKV has been linked to hemorrhagic cystitis, urethral stenosis and transplant nephropathy, while SV40 may be linked to different brain tumors, focal segmental glomerulosclerosis, and infection in pediatric kidney transplant recipients. The laboratory diagnosis is based on cell culture (which is not practical), PCR and serology.

In 1995 BKV was first found to be associated with kidney transplant nephropathy (TN), which can progress to allograft failure. BKV-associated TN can be diagnosed by Papanicoleau staining of decoy cells in urine, which show viral nuclear inclusions, and by quantification of viral load in plasma by quantitative PCR. Low dose cidofovir (which is nephrotoxic) and/or reduction in immunosuppression (which increases the risk of transplant rejection) are the current treatment choices. BKV has also been found in hemorrhagic cystitis in bone marrow or hematopoietic stem cell transplant recipients by monitoring BKV in urine. The relevance of viremia and a BKV real-time assay are presently under investigation and development, respectively.

The Philip A. Hanff Memorial Clinical Case Presentations and discussions were chaired by **Richard L. Hodinka, Ph.D. (RLH)**, Children's Hospital of Philadelphia & University of Pennsylvania School of Medicine, Philadelphia, PA; other contributors were **Marie L. Landry MD, (MLL)**, Yale University School of Medicine, New Haven, CT and **Angela Caliendo, MD, Ph.D. (AC)** Emory University School of Medicine and Emory University Hospital, Atlanta, GA. Six case reports were chosen for discussion:

(1) a 26-year-old male presenting 19 days after tick bite;
(2) a 19-year-old female at 33 weeks of gestation presenting with abdominal pain, fever, nausea and vomiting who had to undergo an emergency Caesarian section due to fetal distress at day 8 in the hospital;
(3) a 13-year-old boy with aplastic anemia and a T-cell depleted mismatched stem cell transplant from his sister presented with persistent cough, two months later with weight loss, chest pain, nausea and vomiting and was tachypneic;
(4) a 14-year-old boy with sickle cell disease, receiving monthly red cell transfusions for secondary stroke prevention, presented with fever and a mild scleral icterus;
(5) a 56-year-old man, who traveled to Vladivostock (Russia) presented with respiratory illness while in Russia and 1 week after his return had severe headache, a day later become confused and had incoherent speech, developed fever, nausea and vomiting;
(6) a 7-month-old boy developed nasal discharge, cough and was fussy, presented with fever and tachycardia with a pulse of 188.

Presentations and discussion took place over two hours, and the authors believe these could not be adequately covered here. The full session is included in the audio CD-ROM described above. The audience and the panel of presenters interactively discussed different potential pathogens, various tests, test methods and therapies, and tried to find a consensus or a "state-of-the-art" procedure finally leading to the diagnosis.

Robert Doms, M.D., Ph.D. (RD), University of Pennsylvania, Philadelphia, PA described progress toward development of entry inhibitors, a new type of drug against HIV. The high replication rate and high transcription error rate that characterize HIV make it a moving target for drug development: statistically, every base in the viral genome mutates to every other possible base every day in an untreated individual. Current drugs target one of two viral enzymes, reverse transcriptase or protease, and one fusion inhibitor was recently approved. Integrase, the third viral enzyme, has been a difficult target. HIV is an enveloped virus, and entry into cells for these involves attachment, receptor engagement, trigger events (pH-dependent or -independent), conformational change and membrane fusion. The trigger event for HIV is its pH-independent engagement with a co-receptor. HIV's receptors are CD4 and co-receptors CCR5 (early) or CXCR4 (later); the switch by the virus from CCR5 to CXCR4 is associated with accelerated disease progression. There is a Δ32-CCR5 polymorphism that prevents HIV entry into cells and protects homozygotes against HIV infection. This suggests that CCR5 is a good drug target, and inhibitors against it are under development.

Opportunities for HIV entry inhibition are being exploited at four points along the pathway: blocking of the native envelope protein trimer; blocking of the CD4 binding step; blocking of the co-receptor binding; and blocking of the 6-helix bundle formation or membrane fusion. Enfuvirtide is a peptide fusion inhibitor that works at this last step. The addition of entry inhibitors to the list of treatment options means that viral phenotype becomes important; in addition to how much virus, clinicians will need to know what kind of virus is present, i.e., which co-receptors does the virus use? Researchers have also observed that different virus strains and cells from different donors each lead to a much larger variability in potency of entry inhibitors 2–4 orders of magnitude) than the variability seen with other types of HIV drugs (2- to

4-fold). This variation, too, should lead to additional testing requirements when these drugs are available for clinical use. A case could be made that patients taking Enfuvirtide should be genotyped for the CCR5 polymorphism, for instance.

RD reviewed mechanism of action studies and sensitivity data for Enfuvirtide and several candidate entry inhibitor drugs, describing virus and host cell factors that affect drug sensitivity and the practical implications of these. Discussion points included the fact that chemokines that are the natural ligands for CCR5 have some protective effect against HIV disease progression, that CXCR4 viruses are less prevalent than CCR5 for reasons that are mysterious, that CD4 expression levels are not infection-rate limiting, but CCR5 levels are, that patients do not make antibody to Enfuvirtide despite being exposed to large amounts of it, that in the presence of CCR5 inhibitors, HIV is evolving to use CCR5 in a different way rather than to use CXCR4 instead, that if the switch from co-receptor use from CCR5 to CXCR4 does take place, it will kill the usefulness of fusion inhibitors, and that entry inhibitors may find application against other enveloped viruses that have pH-independent trigger events.

Kenneth Berns MD, Ph.D. (KB), University of Florida Medical Center, Gainesville, FL reported on Adeno-associated virus (AAV) as a vehicle for gene therapy, beginning with the point that unlike others, this is a potentially useful virus rather than a pathogen. In fact, although 90% of people have antibodies to AAV, it causes no known disease. AAV belongs to the family of the parvoviridae and needs another helper virus, either Adenovirus or any Herpes virus, for replication. The linear 4.7 kb ssDNA encodes 2 ORFs (open reading frames) that result in expression of 4 regulatory proteins in addition to 3 structural proteins encoded in another large ORF. The genes are flanked by inverted terminal repeats (145 bases long). AAV produces progeny viruses in the presence of helper viruses, while in their absence it replicates with cell division. The virus persists for years and may have a protective role in women with cervical cancer.

AAV is the only virus known to integrate into host DNA site-specifically, at a 30 nucleotide stretch on human chromosome 19 (19q13.4), and it is this property that is being exploited for gene therapy. When AAV is used as a vector to introduce a new gene, the internal viral sequences are replaced by the new target gene with appropriate regulatory sequences, while the original terminal end sequences remain. These sequences are required for packaging into the viral coat, for stabilization of the DNA within the cell and for the generation of double-stranded DNA. When packaged, the size of the transgene may not exceed 4.5 kb. AAV vectors have been tested in a variety of model systems and in a limited number of clinical trials involving patients with cystic fibrosis and Factor IX deficiency. KB reported on AAV gene therapy involving diseases of the eye (such as age-related macular degeneration (AMD) and diabetic retinopathy) and the use of AAV vectors in a mouse model.

Frederick G. Hayden, Ph.D. (FH), University of Virginia, Charlottesville, VA, discussed intranasal vaccines for influenza prevention in children. The master donor strain containing the attenuating mutations for CAIV (cold-adapted influenza vaccine) was developed in 1967. The virus is live-attenuated, cold-adapted (25 °C), and temperature sensitive. Above 30 °C it shows a 100-fold or greater reduction in replication. Combining the donor virus with HA and NA gene segments from wildtype antigenic variants results in CAIV. Upon application of CAIV, nasal IgG is elicited as well as a systemic CTL influenza-specific immune response. Two doses are required. FH reported trials with children (aged 15–71 months), COPD (chronic obstructive pulmonary disease) patients, and working age and elderly adults. For seronegative children, the 50% infectious dose for children was 10^4 pfu/child and up to 10^7 pfu/strain could be tolerated. Shedding is as low as 10^3 pfu/mL for about 10 days; person-to-person transmission was rarely observed. Nasal and throat swab specimen testing yielded influenza virus unchanged from the vaccine virus, both genotypically and phenotypically. CAIV shedding in healthy adults, HIV patients or patients with COPD was maximum 50% and did not last longer than 1 week. From another study of almost 10,000 children (aged 1–17 years), adverse side effects (such as acute respiratory, asthma, systemic bacterial and acute gastroenteritis) were not significant as compared to the placebo group. While rhinorrhea was frequently observed (in 15–30%), fever was found only in a small percentage (<5%) within 10 days after vaccination. Comparable results were observed also in vaccinated adults. FH also covered the safety and efficacy aspects in immunosuppressed individuals from contacts with CAIV-T (trial) vaccinated health care workers. Low titers of recoverable virus were detected ($<3.0 \log_{10}$ $TCID_{50}$/mL) in children. The HID_{50} (human infectious dose) for seronsusceptibles was 2.5–4.5 $\log_{10}$ $TCID_{50}$/mL for children and 5.0–6.6 $\log_{10}$ $TCID_{50}$/mL for adults. From CAIV trials in South Africa with 3242 ambulatory older adults (60–97 years), the efficacy against culture-positive influenza was 53% against A/Panama/2007/99, (H3N2), and not effective against B/Sichuan/397/00, resulting in overall efficacies of 42%. FH reported that supplemental CAIV in COPD patients did not add significant efficacy. From a phase III trial with trivalent CAIV (H1N1, H3N2, B, two doses intranasal spray, 8 weeks apart) in 1602 children (15 months to 16 years) the protective efficacy was very high, 95% for H3N2 and 91% for B after one year. These data and other studies presented led to the conclusions that CAIV is easy to administer (nasal spray versus injection); it induces IgA, serum IgG and a specific CTL response; the efficacies are comparable to injected vaccines in adults (possibly superior to trivalent inactivated vaccines in young children), while its use is limited by the U.S. FDA's approval of CAIV for healthy adults only (aged 5–49 years), by the high maintenance costs (frozen vaccine, cold chain) and by the transmission risk. Several other formulations for inactivated intranasal vaccines, such as packaging in liposomes, proteosomes and carbohydrate biopolymers are currently under investigation.

Peter Strebel, MD (PS), Centers for Disease Control and Prevention, Atlanta, GA, described continuing efforts in measles eradication. The measles burden was estimated by the WHO in 2002 to be 610,000 deaths. There are four fundamental criteria for a disease eradication program:

(1) humans are critical for transmission;
(2) sensitive and specific diagnostics must be available;
(3) effective intervention must be possible;
(4) it must be possible to demonstrate prolonged interruption in a large geographic area. For measles, all these criteria are met. Humans are the only major reservoir for the measles virus, and population groups of greater than several hundred thousand are required to sustain transmission. Serologic assays with high sensitivity and specificity for measles IgM exist, and the virus can be isolated and identified in the laboratory. Vaccines exist that are highly effective in developed countries, and strategies have been developed for intervention and surveillance to demonstrate progress. Measles eradication should be achieved in Europe by 2007 and in Africa by 2010, while in America eradication was achieved in 2000. The measles elimination strategy in the US began in 1966 and aimed for high vaccine coverage at age one year, high coverage at school entry, careful surveillance and aggressive outbreak control. In 1998 there were 100 cases in the U.S.; 34 were imported, 33 were linked to imported cases, and 34 were unlinked. In Africa, measles mortality was significantly reduced in the years 2001–2003 by immunization programs that vaccinated 112 million children and averted an estimated 170,000 deaths annually. In summary, major progress has been made: measles has been essentially eliminated in the Americas, and the program is on track for a 50% reduction in mortality worldwide. The goal for worldwide eradication is by 2010; however, polio eradication is a higher priority.

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