



# IAVI Report

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## Clues from CROI

*The 15th annual meeting had its share of sobering news from clinical trials, but researchers also reported many exciting advances*

by **Andreas von Bubnoff**

“Triumph and defeat, success and failure—that’s the theme for this conference.” At least that is how conference vice chair John Mellors of the University of Pittsburgh summed up the 15th Conference on Retroviruses and Opportunistic Infections (CROI) held in Boston February 3-6.

Mario Stevenson of the University of Massachusetts Medical School and conference chair said that the mood coming into this year’s meeting was indeed somber, following the disappointing results of the STEP trial with Merck’s vaccine candidate. This news had an obvious influence on the conference program, which was more focused on AIDS vaccine research and HIV prevention strategies than in previous years. Additional data from multivariate analyses of the STEP trial were presented, as well as news from studies with other HIV prevention methods, including data from additional circumcision studies and the first results from a large randomized clinical trial evaluating whether or not suppression of herpes-simplex virus (HSV)-2 could reduce the risk of HIV infection. There was also a special plenary session devoted to the scientific obstacles that are impeding the discovery of an effective AIDS vaccine.

Stevenson said CROI also showcased “tremendous progress in the basic sciences.” There were a slew of presentations highlighting advances in immunology and biology that open potential avenues of exploration for both HIV prevention and treatment, including the identification of a third cellular antiviral factor, the discovery of a peptide in semen that aids transmission of HIV, as well as a myriad of other fundamental discoveries (see *Scanning the scientific horizon at CROI*, below).

### **One STEP forward...**

Susan Buchbinder of the University of California in San Francisco, and principal investigator of the STEP trial, presented additional results based on multivariate analyses of data from this Phase IIb test-of-concept trial of Merck’s adenovirus serotype 5 (Ad5)-based vaccine candidate, known as MRKAd5. Immunizations were stopped on September 21 last year

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## Scanning the scientific horizon


*An array of fundamental developments in understanding HIV infection and pathogenesis, and the interpretation of immune responses against the virus, headlined this year’s CROI meeting*

by **Richard Jefferys**

Over the past decade, new technologies have emerged that allow researchers to analyze the specificity and function of antigen-specific T-cell responses in exquisite detail. One approach that has been popularized recently by researchers at the Vaccine Research Center (VRC), part of the US

National Institute of Allergy and Infectious Diseases (NIAID), is using multi-parameter flow cytometry to analyze the ability of antigen-specific T cells to perform multiple functions simultaneously—the buzzword that has emerged in the field is “polyfunctionality,” although this should not be taken to mean

that execution of the function automatically equates with *in vivo* efficacy. For CD8<sup>+</sup> T cells, five potential functional markers are typically evaluated: production of the cytokines interleukin (IL)-2, TNF- $\alpha$ , and interferon (INF)- $\gamma$ , and the chemokine MIP-1 $\beta$ , along with expression of a cell surface molecule called

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after an interim analysis by the data safety monitoring board determined that the vaccine did not offer any protection against HIV infection or reduce viral load in individuals who acquired HIV infection. Subsequent analysis of the data indicated there was a trend toward more infections in the vaccinees than in placebo recipients (see *A STEP back?*, *LAVI Report*, September-December 2007).

The first analysis of male volunteers—women were excluded because only one HIV infection occurred in a female volunteer—revealed that vaccinees with higher levels of natural pre-existing Ad5 immunity appeared more susceptible to HIV infection than those with lower Ad5 immunity. The multivariate analyses Buchbinder presented at CROI showed that after adjustment for possible confounding factors such as age, ethnicity, location, and sexual behavior, the apparent increase in susceptibility to HIV infection still appeared to be due to vaccination. “This is still a hypothesis,” Buchbinder cautioned, adding that additional factors needed to be tested such as infection with HSV-2, human leukocyte antigen (HLA) typing, and the possibility of clusters of sexual transmission at certain sites, all of which could have contributed to a spurious result. But, Buchbinder said, “At this point we don’t see any other explanations [apart from the vaccine] for the increased number of infections in the vaccine group.”

Researchers conduct multivariate analyses to analyze several variables simultaneously, while correcting for confounding factors. This type of statistical analysis is used to calculate the potential contribution of each variable on the observed outcome. In this case, researchers used an increasingly complex set of statistical models, each accounting for more variables. Among the vaccinees, the multivariate analyses identified circumcision status as an additional risk factor—vaccinees were over four times more likely than placebo recipients to acquire HIV if they were both uncircumcised and Ad5 seropositive. Buchbinder said it is unclear whether or not the risk of HIV infection was increased for vaccine recipients who had either high levels of Ad5 immunity or were uncircumcised, but not both. In these analyses there was no evidence of enhanced risk of HIV infection in vaccinated volunteers who were circumcised and Ad5 seronegative.

Buchbinder said that the circumcision effect on HIV infection risk seemed to be as strong, if not stronger, than the effect of pre-existing Ad5 immunity. “The interaction for Ad5 was not significant in all the models, but circumcision was significant in most of the models and was of a greater magnitude,” Buchbinder said, referring to the different statistical models used in the analyses. But interpretation of these results is complicated because volunteers were not originally separated based on circumcision status. “What it means is hard to say because we stratified based on Ad5. The circumcision is really a post-hoc analysis and so I think you have to take all of this with a grain of salt.”

Still, she said, “It is possible that this is really from circumcision and not from pre-existing Ad5 immunity.” More than half of the 1,787 men in the analysis were circumcised and 49 were excluded

from the multivariate analyses because their circumcision status was unknown. The role of circumcision in men who have sex with men—the population in the STEP trial—is unclear and is obviously dependent on whether or not the individuals were the receptive or insertive partner during anal sex. “If you are the receptive partner, obviously your foreskin doesn’t matter,” said Michael Robertson of Merck. “We are looking at those kinds of associations as well, but right now we don’t have a clear understanding.”

Robertson presented results of an ongoing characterization of the immune responses induced by MRKAd5 in STEP trial volunteers. Analysis of peripheral blood mononuclear cells (PBMCs) collected at week 30, four weeks after the second vaccination, showed no difference in the potency or breadth of immune responses to MRKAd5 in the volunteers who later became infected with HIV, compared with those who remained uninfected. “It was not [that] the people that became infected were just non-responders to the vaccine,” Robertson said.

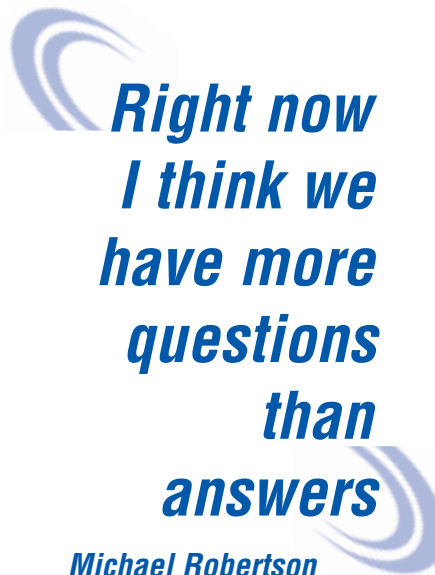
One hypothesis to explain the trend in higher susceptibility to HIV infection among vaccine recipients is that the vaccine might have induced activated T cells, either in response to the vector or the HIV immunogens, and therefore provided additional targets for HIV. But for now this hypothesis does not seem to be supported by the data. “It doesn’t match what we observed in the study,” said Robertson, because T-cell responses to the Ad5 vector, as well as to the HIV inserts were *higher* in people with the *lower* pre-existing Ad5 immunity.

Robertson said there is, however, an important caveat. “We are only looking in the peripheral blood,” he said. “Maybe you are activating cells that don’t stay in the blood but traffic to mucosal sites. Maybe we don’t see an association because we are looking in the wrong place.” He said one biologically plausible hypothesis is that activated cells move to mucosal sites such as the foreskin. “So if you are circumcised you have less targets to get infected,” added Robertson. “It’s biologically plausible but pure speculation at this point.”

Robertson said it would be of interest to check immune responses at mucosal sites, but there are only a few mucosal samples from STEP trial volunteers, such as semen samples, which were collected from individuals after infection. “We don’t have samples just after vaccination in uninfected people,” Robertson said. So for now, there is still no clear explanation for why there was an increased risk of HIV infection in people with high pre-existing Ad5 immunity, Robertson said. “Right now I think we have more questions than answers.”

#### **Extended benefits of circumcision**

In heterosexual men, the protective effect of circumcision has been firmly established by three randomized, controlled clinical trials, which show that surgical removal of the foreskin reduces the risk of HIV acquisition by approximately 60% (see *Vaccine Briefs*, this issue). Additional studies were presented at CROI that looked at the effect of male circumcision on the acquisition of HSV-2, on transmission of HIV from infected men to their female partners, and on acquisition of sexually-transmitted infections (STIs) in female partners.



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**Michael Robertson**

## Pleas for a back-to-basics approach

In 2004, after VaxGen's AIDS vaccine candidate failed in a Phase III clinical trial, CROI featured a session titled, "HIV Vaccines: Clinical Trial Results Call for More Basic Science." The session closed with a talk by Ron Desrosiers of the New England AIDS Research Primate Center: "Why an HIV-1 vaccine is not currently within our grasp." Four years later, the failure of Merck's Ad5-based candidate provided the conference and Desrosiers with an opportunity to revisit this topic. His message was followed by a presentation from Neal Nathanson of the University of Pennsylvania titled, "AIDS Vaccine at the Crossroads." Mario Stevenson, the conference chair, said both speakers were invited to discuss going "back to basics" in vaccine research because "the CROI committee wanted to deliver this message in stereo."

Desrosiers started by asking whether an HIV vaccine is feasible and, as was the case four years ago, he thinks the answer is no. He stated that the naturally occurring immune response to HIV typically fails to control viral replication and does not reliably protect against superinfection with other HIV strains. Desrosiers also cited the daunting diversity of HIV and emphasized that what constitutes a protective immune response against HIV remains unknown.

Researchers, including Desrosiers, have tried to gain information on what type of immune responses are protective by conducting challenge studies in nonhuman primates with simian immunodeficiency virus (SIV). Desrosiers said the best results obtained so far have involved meager viral load reductions of 1-1.5 logs in rhesus macaques challenged with SIV—even under idealized circumstances in which the vaccine and challenge strains are genetically matched. A macaque study with Merck's Ad5 construct encoding SIV Gag failed to protect any animal from infection with SIVmac239 and also had no effect on post-infection viral loads (*J. Virol.* **79**, 15547, 2005). Based on this now infamous study, Desrosiers declared that the failure of MRKAd5 was predictable. He also believes that no other T cell-based vaccine candidate currently being investigated has a "reasonable chance" of demonstrating efficacy.

The negative results of the STEP trial may also erode funding for both clinical trials and basic science, according to Desrosiers. He also said potential volunteers may be alienated and unwilling to enroll in future trials if they hear news of more unsuccessful candidates. Although success of a particular approach cannot be fully assessed without conducting some type of efficacy trial in human volunteers, Desrosiers advised the field to be more selective about which candidates to evaluate in clinical trials and advance only those that have shown significant promise in the SIV model.

Saving perhaps the most controversial aspect of his talk for last,

Aaron Tobian of Johns Hopkins University reported results from a randomized trial in Rakai, Uganda, that enrolled over 3,500 uncircumcised men who were both HIV and HSV-2 uninfected. Half of them were randomly assigned to immediate circumcision, while the other half were offered circumcision at the conclusion of the trial. After two years of follow up, researchers observed that circumcision reduced the risk of acquiring HSV-2 infection by almost 25%. Several observational studies have supported the role of HSV-2 infection in aiding HIV transmission. "This might be part of the reason male circumcision decreases HIV acquisition," Tobian said.

More than 1,600 HIV-uninfected female partners of men in the first trial were also followed for 12 months to determine the effect male circumcision had on the acquisition of vaginal diseases.

Desrosiers asked, "Has the NIH lost its way?" He explained that focusing on clinical development is a departure from the NIH's traditional role of supporting basic science and leaving industry to explore promising leads in costly clinical trials. Desrosiers acknowledged that so far the pharmaceutical industry has been reluctant to focus on AIDS vaccine development but he claimed, "When an HIV vaccine does become feasible, pharma will jump on product development, manufacturing, and clinical testing." To emphasize what he believes are the skewed priorities of the current NIH vaccine portfolio, Desrosiers showed a slide indicating that 33% of NIH funding supports clinical trials. In 2007, he said, the NIH spent US\$189 million on AIDS vaccine clinical trials.

Desrosiers asserted that instead funding should support efforts to understand the induction of broadly neutralizing antibodies against HIV, the mechanism of protection with live attenuated SIV vaccines in nonhuman primates, and the causes of long-term nonprogression in humans. He also urged comparative testing of vaccine candidates in the SIV/macaque model and consideration of novel ideas, such as the hybrid vaccine/gene therapy approach developed by Phil Johnson of the Children's Hospital of Philadelphia that uses an adeno-associated virus (AAV) vector to persistently express monoclonal neutralizing antibodies (see *IAVI Report* April-June, 2005). Desrosiers described the as-yet-unpublished results of Johnson's SIV challenge studies as "dynamite."

Nathanson followed by echoing Desrosiers's call for more basic discovery, starting with the quest to identify neutralizing antibodies against HIV. Nathanson's hypothesis is that there may be a finite number of sites in HIV's envelope that can mutate to avoid antibodies targeting the CD4 binding site. He therefore suggested that a basic research priority should be answering the question of whether all possible mutations in the CD4 binding site of HIV's envelope can be characterized and targeted by a multivalent immunogen.

Shifting gears to monkey models, Nathanson diverged somewhat from Desrosiers' view that SIV infection of macaques is the *sin qua non* for vaccine research. Instead, he stressed the importance of evaluating multiple different challenge models, with varying levels of stringency (heterologous versus homologous challenges using a variety of SIV strains and hybrid SIV/HIV isolates known as SHIV) to obtain a comprehensive view of a vaccine candidate's potential. Nathanson closed by concurring with Desrosiers and suggesting that the NIH's vaccine research portfolio needs to be reassessed. The format for this CROI session did not allow time for questions or comments at the end of the talks by Desrosiers and Nathanson. —R.J.

Researchers found that the female partners of circumcised men had an approximately 25% lower risk of acquiring genital ulcer disease, an almost 50% lower risk of acquiring *Trichomonas vaginalis*, and an almost 20% lower risk of acquiring bacterial vaginosis, compared with female partners of uncircumcised men. These results are consistent with previous observational studies, Tobian said. A possible explanation is that in uncircumcised men, the moist cavity created by the foreskin may favor the survival of bacteria.

Knowing the effects of circumcision in HIV-infected men is also important, according to Maria Wawer of Johns Hopkins University. She says that in some communities being uncircumcised may stigmatize men as being HIV infected. To avoid this, some HIV-infected men may seek circumcision. Wawer reported results from

another randomized trial in Rakai, Uganda that enrolled discordant couples—HIV-infected men with uninfected female partners—to study the effects of circumcision in HIV-infected men and on HIV transmission rates to women. In this trial, 93 couples were enrolled in the intervention arm, in which the male received immediate circumcision, and 68 couples were enrolled in the control arm. Men in the control group were offered circumcision after the trial was complete.

After two years of follow up, researchers found that circumcision did offer some benefit to HIV-infected men—rates of genital ulcers were reduced by about 50% in circumcised trial participants compared to uncircumcised controls. But circumcision had no effect on HIV transmission rates to the female partners of HIV-infected, circumcised participants. This result was “unexpected and disappointing,” Wawer said. “In previous observational data we had seen lower HIV rates in women married to HIV-positive circumcised men compared with HIV-positive uncircumcised men.”

Researchers suggested the women were not protected because some of the couples resumed sex too soon after surgery. “If the males resume intercourse early after circumcision, before the wound is fully healed, there might be increased transmission,” said Wawer. In the 18 couples who reported resuming sex before the wound healed completely, 27% of the female partners were infected with HIV in the first six months of the study, compared to only 9.5% of female partners who were infected after waiting to resume sexual activity. Wawer said it is very important that people not resume sex in the early postoperative period, even if they are not HIV infected. “In our trial of negative men the protective effects of circumcision became significant and apparent only after the six-month follow-up period,” she said, referring to an earlier trial of male circumcision (*Lancet* 369, 657, 2007).

### **Stopping HSV doesn't stop HIV**

More sobering news on the HIV prevention front came from Connie Celum of the University of Washington in Seattle, who reported the results of a randomized clinical trial (HPTN 039) assessing whether or not administering suppressive therapy for HSV-2 could reduce HIV acquisition rates.

The trial enrolled 3,277 volunteers who were infected with HSV-2 but not HIV. Volunteers included men who have sex with men in the US and Peru and heterosexual women at sites in Zimbabwe, Zambia, and South Africa. All participants randomized to the intervention group received a twice-daily 400 mg dose of acyclovir, an antiviral drug used in the treatment of HSV-2 infection, which is the cause of genital herpes. After 18 months, there was no difference in the number of new HIV infections between the group that received acyclovir and the control group. Several observational studies have shown that HSV-2 infection increases susceptibility to HIV by two- to three-fold, so this was an unanticipated result. “Many people thought this would be a slam dunk,” said Celum. Taking acyclovir did reduce the incidence of genital ulcers by 37%, but even this was much lower than what was observed in previous studies.

“Why didn't we have an effect on HIV at all?” Celum asked. She thinks it is unlikely that HSV-2 is not a risk factor for HIV, given the epidemiological data that suggests otherwise. Adherence is one factor that could have influenced the results. Reported levels of adherence to acyclovir were high in the study, but Celum said it could have been overestimated since it was based on self-reported behavior. The less-than-expected reduction in occurrence of genital ulcers also varied geographically, suggesting biological reasons may account for why researchers did not see any effect on HIV infection rates, Celum said. For example, there may be differences in how the drug was metabolized or in susceptibility of the virus to the drug.

### **Tethering the virus**

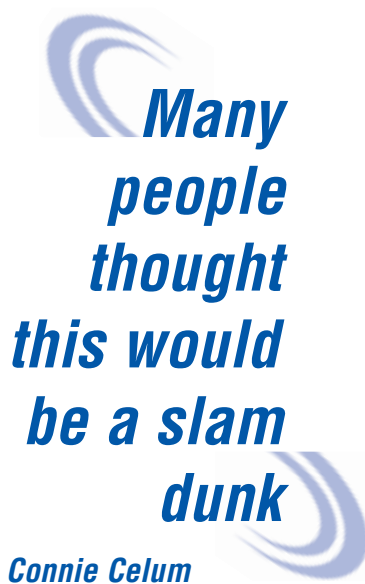
A basic science finding that caused a stir was the recent discovery by Paul Bieniasz' group at Rockefeller University, and independently by John Guatelli and colleagues at the University of California in San Diego, of the existence of another naturally-occurring antiviral factor in human cells (*Nature* 451, 425, 2008). This newly-discovered molecule named tetherin is the third cellular antiviral factor that has been identified to date, in addition to TRIM5 $\alpha$  and APOBEC3G (see *Guardian of the genome*, *LAVI Report*, April-June 2005). As the name suggests, tetherin keeps newly made HIV particles tethered to the surface of an infected cell after budding. “The cell behaves altruistically,” Bieniasz said. “It itself is doomed, but other parts of the body might be saved by this act of the cell to retain the virus to its surface.”

Researchers have known for a while that HIV lacking its accessory protein Vpu remained stuck to the surface when infecting a certain subset of cells dubbed non-permissive, but had no problem escaping other, so-called permissive cells. While it was unclear what kept the HIV particles tethered to the membrane of non-permissive cells, Bieniasz and colleagues had shown that this property could be induced by treating permissive cells with IFN- $\alpha$ .

“This gave us a handle to try to identify genes that might be responsible,” Bieniasz said. His lab conducted a microarray experiment that compared expression levels of IFN- $\alpha$ -induced genes in permissive and non-permissive cells and looked for a cell surface or secreted protein. They identified tetherin, a membrane protein that co-localizes with accumulating virus particles on the cell surface, as the molecule responsible for the non-permissive phenotype. “[This is] a brand new mechanism which many of us never suspected before,” said John Coffin of Tufts University, who was not part of the study. Researchers have not yet figured out exactly how tetherin actually performs this function or how Vpu counteracts its effect, Bieniasz said, but by inactivating Vpu, it may be possible to mobilize this defense against HIV. “Hopefully pharmaceutical companies will take this on,” Bieniasz added.

### **Enhancer protein**

Another study described the discovery of natural compounds, derived from human body fluids, that inhibit or enhance HIV infection. Frank Kirchhoff of the University of Ulm, in collaboration with



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**Connie Celum**




Wolf-Georg Forssmann of Hannover Medical School, have established peptide libraries from human body fluids, which have led to the discovery of a natural enhancer of HIV infection.

Kirchhoff's lab isolated an array of peptides from human semen, a logical place to look for factors affecting the efficiency of HIV infection, Kirchhoff said. From this peptide library, they identified an abundant peptide called prostatic acidic phosphatase (PAP). Initial experiments failed to show any effect of PAP fragments on HIV infection, but after an overnight incubation period, it was discovered that PAP could enhance HIV infection. "At some point we left it overnight and did the experiment the next day and it turned out it was actually becoming active," said Kirchhoff.

His group found that PAP fragments form amyloid-like fibrils that can capture HIV virions and promote their attachment to target cells, dramatically enhancing HIV infection in cultured cells (*Cell* **131**, 1059, 2007). "When you incubate the fibrils with virus particles," Kirchhoff said, "they all bind after a few minutes, probably by just electrostatic interaction." This binding is independent of the

sequence of HIV's Env protein. They also bind to the target cells with high efficiency, he said. The fibrils enhanced the infection rate of diluted HIV added to cultured PBMCs by 100,000-fold. "To be honest I did not believe the data, so we repeated this over and over and the results were pretty consistent," added Kirchhoff. The fibrils also enhanced HIV infection in relevant human samples such as *ex vivo* cervical tissue, and *in vivo* in transgenic rats expressing human transgenes.

Kirchhoff said PAP could be a new target for blocking HIV transmission. "Given that the viral dose during sexual intercourse is normally sub-infectious, blocking an enhancing activity should have a direct impact on the rate of HIV transmission," said Kirchhoff. He also suggested that a 10- to 20-fold higher dose of microbicides might be required to overcome the fibroid activity of PAP. "When we test microbicides with or without semen and the enhancer we need much higher doses to block the virus," Kirchhoff said. The fibrils may also enhance other types of infections; his group has already observed this with HSV, hepatitis C virus, and Chlamydia. 

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CD107a, which is a marker for cytotoxic (cell-killing) potential. Data is then reported based on the number of these markers an individual T cell displays. The growing enthusiasm for this approach is largely driven by data demonstrating an association between polyfunctional HIV-specific T cells and control of viral replication and non-progression in infected individuals (*Blood* **107**, 4781, 2006).

But application of new technologies can sometimes lead to challenges in data interpretation. At CROI, Hendrik Streeck of the Partners AIDS Research Center in Boston delivered a cautionary tale about interpreting data on T cell polyfunctionality in HIV infection. To date, Streeck noted, studies have evaluated polyfunctional CD8<sup>+</sup> T-cell responses to multiple pooled peptides, as opposed to individual epitopes. In a cohort of individuals with acute HIV infection, Streeck decided to take a different tack. He analyzed the profiles of CD8<sup>+</sup> T cells targeting known HIV epitopes and then looked at the relevant genetic sequence of the virus for any evidence of immune escape mutations. Streeck's analysis revealed that CD8<sup>+</sup> T cells with putatively broader functionality (displaying three or more of the above-listed functional markers) were more likely to be targeting epitopes in which escape mutations had developed. These CD8<sup>+</sup> T cells were therefore not being stimulated because the epitope they were targeting was no longer present in the virus. In contrast, epitope-specific CD8<sup>+</sup> T cells displaying only one of the above functional markers were targeting epitopes that had not escaped. Streeck emphasized that these data do not necessarily mean that all polyfunctional responses are unimportant, but rather supports careful interpretation of studies employing this approach for evaluating T-cell responses.

### Surveying the genome

David Goldstein from Duke University and the Center for HIV/AIDS Vaccine Immunology (CHAVI) provided an update session on ongoing efforts to uncover host genetic influences on control of HIV replication and disease progression, following work published last year with collaborator Amalio Telenti of the University of Lausanne (*Science* **317**, 944, 2007). These studies exploit a new method of genetic analyses that allows investigators to assess the impact of common variations in the human genome—called single nucleotide polymorphisms or SNPs—on the outcome of interest.

Goldstein initially focused on viral load set points, and in a study of 486 individuals, three associations were uncovered that remained significant, even after correcting for the staggering half a million different SNP analyses conducted. The first association, which accounted for close to 10% of the variation in viral load set point, was with an SNP located in the HLA complex P5 (HCP5) gene. This gene is known to be linked to the HLA B\*5701 allele, which is a well-described correlate of viral control and non-progression in HIV-infected individuals, suggesting that the HCP5 SNP mediates its effect by affecting the HLA B\*5701 allele in some way. The HLA B\*5701 allele encodes a CD8<sup>+</sup> T-cell receptor reported to be particularly adept at recognizing HIV epitopes. However, in the original *Science* paper, Goldstein and colleagues noted that HCP5 is an endogenous retroviral element and so could conceivably have direct effects on HIV replication, perhaps via an anti-sense mechanism. At CROI, Goldstein reported that extensive *in vitro* studies failed to demonstrate any inhibition of HIV, leading him to conclude that an independent effect of HCP5 is unlikely. From the perspective of vaccines this may be somewhat encouraging, as it suggests that the association between the HCP5 SNP and viral load set point is likely related to the CD8<sup>+</sup> T-cell response to HIV.

The second association that emerged was with an SNP in the gene encoding HLA-C, which is also involved in the presentation of epitopes to CD8<sup>+</sup> T cells. As background, thousands of HLA molecules adorn every cell in the human body, with the exception of red blood cells, and their function is to display fragments of proteins (epitopes) lifted from inside the cell to passing CD8<sup>+</sup> T cells. Should any of these epitopes be recognized as foreign (for example if the cell is infected with HIV), a CD8<sup>+</sup> T cell can release enzymes that kill the infected cell. The HLA molecules that interact with CD8<sup>+</sup> T cells are called class I, and within this class there are three main sub-families: HLA-A, HLA-B, and HLA-C. Studies have shown that cells express far more HLA-A and HLA-B molecules than HLA-C, so presentation of HIV epitopes by HLA-C has generally been less studied. Goldstein's hypothesis is that this second SNP may be associated with increased expression of HLA-C on cells, potentially facilitating enhanced CD8<sup>+</sup> T-cell recognition—an attractive hypothesis given that HIV's Nef protein is known to downregulate HLA-B molecules from the surface of infected cells as a

## A twist in the tale

Robert “Chip” Schooley of the University of California in San Diego presented data from a therapeutic trial of a prototype of Merck’s Ad5-based AIDS vaccine candidate encoding only HIV Gag. Given the much-publicized failure of the final vaccine construct (which encoded HIV Gag, Pol, and Nef) in a large preventive trial, Schooley’s presentation offered an odd twist.

The therapeutic vaccine trial enrolled 114 HIV-infected individuals on antiretroviral therapy, who had CD4<sup>+</sup> T-cell counts over 500 and viral loads less than 500 copies/ml for at least two years prior, and at or below 50 copies/ml at study entry. Individuals with high anti-Ad5 antibody titers (over 1:200) were excluded. Participants received three doses of either the Merck prototype vaccine or placebo. Three months after the final immunization, antiretroviral therapy was interrupted for 16 weeks. During this period therapy was reinitiated if certain safety thresholds were breached or at a participant’s request. The co-primary endpoints of the trial were the area under the curve (AUC) of all viral load measurements during the 16-week treatment interruption—AUC is a method for calculating how much virus each

individual was exposed to as a function of time—and viral load set point (the average of viral load levels at weeks 12 and 16 post-interruption).

Reviewing the results, Schooley reported that a meager reduction in the viral load AUC was observed in vaccinees compared to placebo recipients but statistically this result only represented a strong trend. HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses appeared to be bolstered by vaccination and, interestingly, there was a statistically significant inverse correlation between the magnitude of the HIV-specific CD4<sup>+</sup> T-cell response, as measured by IFN- $\gamma$  ELISPOT assay, prior to interruption and the post-interruption viral load set point. No correlation was seen with the HIV-specific CD8<sup>+</sup> T-cell response. Schooley concluded that the data support pursuing the goal of enhancing viral load control immunologically, but he believes there is not an immunogen available that is potent enough to improve upon these results. Immunologist Mike Lederman, the vice chair of the protocol for this study, was a little more sanguine. “With one antigen derived from a single HIV sequence, I was encouraged we saw any positive signal at all.” —R.J.

means of immune escape. He has found some support for this idea in published databases but, so far, efforts to independently confirm the association between the SNP and HLA-C expression (by measuring the effect of the SNP on HLA-C messenger RNA levels in cells) have been stymied by unanticipated variation dependent on which HLA-C allele is used as a probe. Goldstein is now collaborating with Andrew McMichael to directly measure HLA-C protein expression on cells of individuals possessing the beneficial SNP.

The third association discussed by Goldstein is with a set of seven SNPs located in or near a pair of more obscure genes dubbed ZNRD1 and RNF39. ZNRD1 encodes a protein involved in RNA transcription. The plausibility of this association was recently buttressed by the finding that ZNRD1 is one of a dizzying array of host proteins needed by HIV to replicate in human cells (*Science* 10 Jan 2008, DOI: 10.1126/Science.1152725). The function of RNF39, however, requires further study. In the case of these SNPs, the stronger association was initially seen with disease progression (defined as time for a CD4<sup>+</sup> T-cell count to decline to below 350 or initiation of antiretroviral treatment) rather than viral load set point. Importantly, however, Goldstein was able to unveil new data from an additional 1,000 HIV-infected individuals and all of the above-described genetic polymorphisms were associated significantly with viral load set point. Based on these additional analyses, he stressed that “these polymorphisms are most certainly real effects that are here to stay.” Further analyses will aim to reveal the functional relationships between the SNPs, relevant gene products, and control of HIV replication.

To give a broader sense of the profound interplay between host genetics and HIV disease progression, Goldstein evaluated the combined effect of these newly discovered polymorphisms in the HCP5, HLA-C, and ZNRD1/RNF39 genes along with two other favorable genetic polymorphisms in the CCR5 and CCR2 genes (CCR5 32 and CCR2 V64I). Possession of one or two favorable mutations in at least four of these genes was associated with a four-times-longer average period before CD4<sup>+</sup> T cells declined to less than 350 (from less than 2 years to more than 8).

Goldstein closed by describing the future plans of his CHAVI team, which include a potentially important evaluation of genetic associations

with the magnitude of antibody responses generated by participants in the efficacy trials of VaxGen’s gp120 vaccine, AIDSVAX. Although the vaccine failed to protect against HIV infection, an association was seen between the magnitude of an individual’s antibody response to the immunogen and susceptibility to HIV infection; participants who generated high levels of antibodies were less likely than placebo recipients to acquire HIV infection, while those who mounted poor antibody responses were more susceptible to HIV than the placebo group. Goldstein’s study could therefore shed additional light on the genetic influences on both the immune response to vaccines and susceptibility to HIV infection, and might even assist researchers in understanding the surprising association between the magnitude of antibody responses to adenovirus and susceptibility to HIV infection observed among placebo recipients in the STEP trial.

### Debating gut depletion

Several years ago, a number of scientists popularized the view that loss of CD4<sup>+</sup> T cells from the gut mucosal tissue in the first few weeks of infection was a catastrophic insult from which the immune system never recovers, and the most important augury of the ultimate development of immunodeficiency. But, as described by Cristian Apetrei in a poster discussion talk titled “HIV Pathogenesis: Viral Blitzkrieg or 10 Years’ War?,” emerging data is prompting many researchers to reconsider this theory.

Apetrei provided examples from several different pathogenic and nonpathogenic models of SIV infection illustrating that the impact on gut CD4<sup>+</sup> T cells is variable and not predictive of disease progression. He also showed an example of a rhesus macaque infected with a pathogenic SIV isolate that progressed to simian AIDS despite experiencing very little acute depletion of gut CD4<sup>+</sup> T cells. Apetrei argued that events during the chronic phase of infection—such as increasing immune activation and anti-SIV antibody responses—are more important drivers of disease progression than the early loss of gut CD4<sup>+</sup> T cells.

Satya Dandekar of the University of California at Davis described results of a new study that approached the issue of gut CD4<sup>+</sup> T-cell depletion from a novel angle by looking at a specific subset of gut CD4<sup>+</sup> T cells rather than the population overall. Dandekar focused in on Th17 (T helper type 17) CD4<sup>+</sup> T cells, a recently discovered

lineage characterized by the production of interleukin-17 (IL-17) that is important in the control of microbial pathogens and yet also associated with some pro-inflammatory autoimmune conditions. In a collaboration with Andreas Baumler's lab, Dandekar conducted studies in rhesus macaques with the microbial pathogen *Salmonella typhimurium*. When this bacteria was injected directly into the gut tissues of macaques, Dandekar observed an upregulation of IL-17. This response was blunted in SIV-infected macaques, while other cytokine responses, such as IFN- $\gamma$ , remained unchanged. In addition, in SIV-infected macaques the *Salmonella* bacteria spread to other tissues, such as the mesenteric lymph node and the spleen. Normally, *Salmonella* infection remains limited to the gut. "The impact of not having an IL-17 response was that [*Salmonella*] disseminated to other organs," Dandekar said. This same phenomenon was also observed in mice experiments. Dandekar said the loss of Th17 CD4<sup>+</sup> T cells in the gut, which leads to incomplete clearance and dissemination of microbial pathogens, may be a "major contributor to the chronic immune activation seen in HIV and SIV infections."

### **Th17 in the spotlight**

Dandekar's talk was a prelude to a triumvirate of similar studies presented at CROI. Mirka Paiardini of Guido Silvestri's laboratory at the University of Pennsylvania described the differential impact of pathogenic and non-pathogenic infections on the Th17 cell population in the gut. Paiardini noted that CD4<sup>+</sup> T cells are now generally characterized as belonging to one of four broad categories: Th1 cells, which primarily produce IFN- $\gamma$  and are important in cell-mediated immunity; Th2 cells, which produce IL-4 and other cytokines important in supporting antibody (humoral) responses; Treg cells which can produce IL-10 and TGF- $\beta$  and have a suppressive, regulatory role; and the newly discovered Th17 cells which mainly produce IL-17 and IL-22 and, as Satya Dandekar highlighted, are important in antimicrobial responses (such as against *klebsiella pneumoniae* and *candida albicans*), as well as being associated with some autoimmune conditions. Paiardini also cited studies indicating that Th17 cells have an important role in tissue repair, promoting epithelial cell proliferation and thus helping maintain the integrity of the mucosal epithelium.


Paiardini's study investigated whether Th17 cells have a role in HIV pathogenesis by comparing responses in HIV-infected and uninfected humans and in non-pathogenic SIV infection of sooty mangabeys, a natural host of SIV. He reported that, in humans, no differences were seen in blood samples, but in samples from the gut, Th17 responses were significantly reduced, even in individuals on prolonged antiretroviral therapy. Further analysis revealed that about 60% of Th17 cells in the gut expressed the HIV co-receptor CCR5 and it was this population that appeared to be preferentially depleted. A significant association was also seen between levels of immune activation—in both the gut and the blood—and Th17 cell depletion. Because non-pathogenic SIV infection of sooty mangabeys is associated with a lack of immune activation, Paiardini went on to assess levels of Th17 cells in the gut of these animals, discovering that they remained stable despite the fact that gut CD4<sup>+</sup> T-cell numbers overall were significantly lower than in uninfected sooty mangabeys. He also showed that maintenance of gut Th17 cells was associated with a preserved mucosal epithelium, suggesting that this may be an important factor in protecting sooty mangabeys from the pathogenic consequences of SIV infection. This would be consistent with the idea, proposed by

Danny Douek and Jason Brechley of the US National Institutes of Health, that translocation of bacteria across damaged mucosal epithelium contributes to systemic immune activation in HIV infection.

The subsequent presentation from Valentina Cecchinato of the National Cancer Institute provided additional support for Paiardini's hypothesis. Cecchinato showed that depletion of Th17 cells from the gut of rhesus macaques is a significant predictor of disease progression. Cecchinato also showed that asymptomatic "elite controller" macaques that control SIV replication maintain gut Th17 cell levels.

The third Th17 talk was delivered by David Favre of the University of California in San Francisco (UCSF). Favre reported results from studies comparing infection of pigtailed macaques and African green monkeys (AGMs) with the same AGM-derived SIV isolate, showing that while immune activation was persistent in the former species, it is transient in the latter. Favre looked at Th17 cells based on the hypothesis that these pro-inflammatory cells may contribute to immune activation in pigtailed macaques but, like the prior studies, found the opposite. Th17 cells were preserved in AGMs, but depleted from all tissues studied in the pigtailed macaques, including the colon. Since previous studies have suggested that a balance between Th17 and Treg cells may be important, Favre went on to look at Treg cells in the two monkey species, finding that the curtailment of immune activation in AGMs was associated with an early increase in Tregs in both the lymph nodes and blood. This effect was sustained over time. In contrast, only a transient increase in Tregs was seen in pigtailed macaques and it occurred much later in the course of disease. Favre suggested that the maintenance of the balance between Th17 and Treg cells may be particularly important in preventing disease in AGMs (a species that, like sooty mangabeys, does not develop immunodeficiency as a result of SIV infection). He also showed data from a study conducted in collaboration with Peter Hunt and Steve Deeks, both of UCSF, which found that the degree of imbalance between Th17 and Treg cells in rectal biopsies from HIV-infected people correlated strongly with the level of systemic immune activation.

### **Breaching the vaginal barrier**

Scott McCoombe of Northwestern University presented new findings from imaging studies using fluorescent-labeled HIV to illuminate the early interactions between the virus and the vaginal epithelium. McCoombe showed that virions can penetrate several layers deep into the intact epithelial wall, seemingly through the junctions between epithelial cells which were previously thought too narrow to allow HIV to penetrate—the average virion size is about 80-100 nanometers, whereas the epithelium was thought impervious to particles much above 30nm. McCoombe suggested that this may facilitate heterosexual transmission by allowing the virus to get far enough to be taken up by immune system cells (such as dendritic cells, T cells and macrophages) present beyond the epithelial surface. He also noted that any factors that increase the quantity of immune cells present (such as inflammation) and/or impair the integrity of the epithelium (such as ulcers, trauma, or hormone-related thinning) would greatly increase the risk of HIV transmission. Additional work is ongoing to better understand precisely how HIV penetrates the surface of the intact epithelium. 

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# Conducting HIV prevention research in South Africa

*Challenges of and successes in conducting HIV research in KwaZulu-Natal, the epicenter of the South African HIV epidemic*  
**by Koleka Mlisana, Francois van Loggerenberg, and Salim Karim**

Sub-Saharan Africa is the most severely HIV-affected region of the world and accounts for 63% of global infections<sup>1</sup>. The HIV epidemic in South Africa is characterized by very high prevalence rates, linked to persistently high incidence rates. In 2006, HIV prevalence in pregnant women was 29% nationally, and 39% in KwaZulu-Natal, the worst-affected province. Hence both prevention and treatment are a high priority in this setting.

However, working in these areas also presents significant challenges, including resource constraints and bottlenecks. As additional HIV prevention trials get underway, identification of hurdles and bottlenecks not only in the design, but also in the implementation of running such trials, will be of paramount importance.

South Africa is well positioned to conduct HIV prevention trials, with an established clinical research infrastructure, world-class laboratories, and trained clinical trialists. Recently South Africa was hosting a Phase IIb test-of-concept trial known as HVTN 503, or Phambili, with Merck's AIDS vaccine candidate (MRKAd5). But in September of 2007, immunizations and enrollment were stopped because data from the HVTN 502 companion study—known as the STEP trial—testing the same vaccine candidate showed it was ineffective.

As the field prepares for other Phase IIb test-of-concept and Phase III efficacy trials with vaccines and microbicides, the limitations of conducting research in highly-affected areas need to be taken into consideration. South Africa, which has one of the highest HIV prevalence and incidence rates in the world, yet better resources than most developing countries, is in an advantageous position to shed light on some of these challenges.

## **State of the epidemic**

In South Africa, about 5.5 million people—approximately 19% of the adult population—are currently estimated to be living with HIV<sup>2</sup>. The epidemic has grown explosively. Between 1990-2005, national HIV seroprevalence among antenatal clinic attendees increased from 0.7% to 29.1%<sup>2-4</sup>. The province of KwaZulu-Natal is at the most advanced stage of the epidemic and here antenatal HIV prevalence reached 39% last year.

The majority of HIV infections in this region occur through heterosexual transmission and about two to three times more young women are HIV infected, as compared to young men<sup>1</sup>. In one rural

KwaZulu-Natal district, the HIV prevalence reached almost 50% in 2006 among 20- to 29-year-old women.

Not only are young women in South Africa more severely affected but they also acquire their infections at a much earlier age than men<sup>5</sup>. These striking gender differences have contributed to the rapid growth of the country's HIV epidemic, along with high rates of sexually-transmitted infections (STIs)<sup>6</sup>, the migrant labor system<sup>7</sup>, and high rates of gender-based violence<sup>8</sup>.

Although not unique to South Africa, marginalization and discrimination on the basis of race and/or ethnicity has also played a role in influencing vulnerability to HIV infection<sup>9</sup>.

## **Challenges of establishing a cohort**

Between August 2004 and May 2005, the Centre for the AIDS Programme of Research in South Africa (CAPRISA) established a cohort of HIV-uninfected high-risk women for an acute HIV infection study (CAPRISA 002) that aimed to describe immunological, virological, and clinical characteristics of HIV-1 subtype C acute infection in Durban<sup>10</sup>, and to determine the incidence in a high-risk population. Risk factors such as sex work, STIs, and HIV prevalence have been used in the past<sup>11</sup> as the basis for selecting populations for HIV prevention studies because it is assumed that these populations would also have high HIV incidence rates.

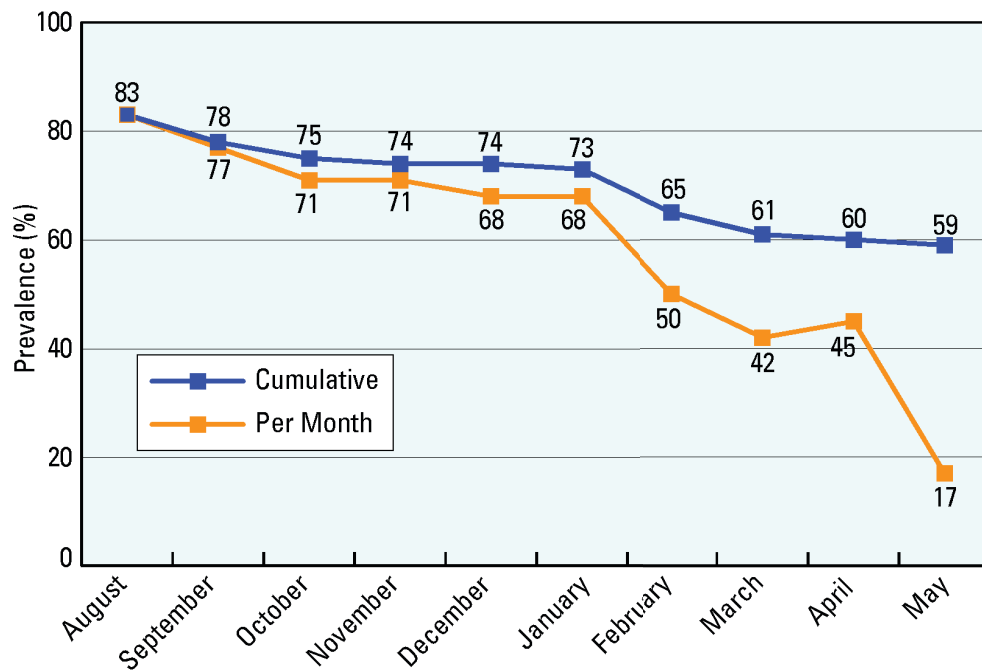
However, these measures are crude and unreliable and at best only hint that there might be high incidence in a highly generalized epidemic such as the one in this region.

The aim of this study was to recruit female sex workers (FSWs). Although women are often reluctant to identify themselves as FSWs, 79% of our cohort consisted of self-identified FSWs when evaluated during behavioral risk assessments.

Establishing HIV-negative cohorts in such high prevalence areas can be a great challenge. In many areas, routine HIV testing is not yet common. Offering HIV testing in preparation for research studies may lead many individuals to undergo HIV testing who otherwise would not have had easy access to this service. Therefore many HIV-infected individuals are often uncovered in the process of trying to identify uninfected trial volunteers. In this sex-worker cohort, the overall HIV prevalence at screening was nearly 60% (95% CI; 55.9%-62.8%). The HIV prevalence varied dramatically over time from 83% in the first

*There are many complications associated with conducting HIV prevention studies in severely-affected regions*





**Figure 1.** HIV prevalence, both per month and cumulatively, in participants screened for enrollment into CAPRISA 002, a Phase I study of acute HIV infection. This graph illustrates the fluctuation in HIV prevalence of potential volunteers for this study, which can greatly impact trial staff.

month of screening to 17% in the last month (Figure 1). This variation indicates how unpredictable the enrollment process can be.

### **The implications of high prevalence**

There are many complications associated with conducting HIV prevention studies in severely affected regions. First, the higher-than-anticipated prevalence can have an indirect impact on clinic staff. They can easily become overwhelmed by having to counsel and follow up such large numbers of HIV-positive patients, who most often are not expecting the diagnosis. It is critical that employees at the clinic, especially counselors, are given an opportunity to be debriefed on the stress associated with this kind of work. Counseling may need to be provided to staff members who become disillusioned by the number of HIV-infected patients they are seeing in their clinics.

Such high prevalence rates can also put pressure on recruitment deadlines and screening numbers in prevention research, and can increase the patient burden at local clinics identified as referral partners for the treatment and management of HIV infection. It is therefore critical to provide support to these clinics in the form of train-

ing, access to medicines, resources, and counseling and support staff, if necessary, to assist them in dealing with a significantly expanded workload. It is also important that memoranda of agreement regarding referrals and the sharing of critical resources are in place prior to screening efforts, and that resources to support and expand existing facilities are part of large grants from the outset.

It is also essential to develop strategies when working with marginalized populations such as FSWs, which are difficult to access. It is often useful to identify networks of peer sex-workers to assist in establishing and maintaining cohorts. Visits to sites often need to be arranged outside regular work hours, at times and places that are convenient for the research participants.

Communication with these volunteers is often challenging because of high mobility and a lack of fixed residential addresses or contact numbers. Mobile telephones, which are widely used in South Africa, have therefore proven to be an extremely useful communication tool. A web-based short message service (SMS) has been used to send pre-approved mobile phone text messages to willing participants and proved to be an effective tool for clinic visit reminders. This is a cost-effective service

**High prevalence rates can also put pressure on recruitment deadlines and screening numbers in prevention research**

and also provides a convenient electronic and paper audit trail. Almost all participants either had their own cell phone, or had a friend who had a cell phone. Mobile telephone contact and SMS messaging were used in the HVTN 503 trial to call trial participants to the clinic to notify them of the trial suspension, and subsequently, to inform them when immunizations were halted.

High mobility and low socioeconomic status of many research participants in this environment can further complicate cohort retention. Fortunately, retention rates for CAPRISA 002 were over 84% after two years, with only 5% of the HIV-negative cohort falling out of contact during the follow-up period. This retention is reassuring for future microbicide and vaccine studies, which could have even greater retention rates in light of the perceived benefits of the intervention to the participants. This retention success underscores the value of having a dedicated network of peer workers and of making a concerted effort to engage the community in the research. Making use of all available contact channels, especially the use of mobile phones, is essential for maintaining follow-up.

In addition to communication and retention, there are other unexpected challenges when working with highly-affected populations. One of these is the high prevalence of domestic violence. An alarming one-third (32.4%) of the women in the CAPRISA 002 cohort either reported or had clinical evidence of abuse and in 70% of cases, the abuser was an intimate sexual partner. Responding to this challenge required establishing relations with available support structures like the Network on Violence Against Women and necessitated specific training for research staff, especially counselors, on domestic violence.

### **Measuring incidence**

HIV incidence measures are essential for monitoring the future trends of the epidemic, for understanding the dynamics of the epidemic, for designing HIV prevention trials, and for estimating the number of new infections—all of which are essential for planning and monitoring interventions. Accurate measurements of incidence are crucial in the face of prevention trials, as HIV seroconversion is critical in endpoint-driven vaccine trials. Statistical power of these trials is dependent on accurate estimates of the incidence rate. Longitudinal cohorts are therefore often the best for incidence measurement, but can be very expensive and time consuming. In the CAPRISA 002 study cohort, the high HIV prevalence was coupled with a remarkably high HIV incidence rate. With 4,784 monthly visits by 245 participants, 28 acute HIV infections were identified for an annual seroincidence rate of 7.2 per 100 person-years (95% CI; 4.5-9.8), despite intense risk-reduction counseling for all participants.

It is important to note for prevention trials testing microbicides or AIDS vaccines that high risk sexual behavior continues despite the best attempts by study staff to provide condoms and risk-reduction counseling. HIV prevention studies will therefore need to ensure that there are comprehensive plans for risk-reduction counseling, especially since HIV infection is often the endpoint for these studies and the community may believe researchers are therefore not motivated to reduce infection rates. It can be confusing for communities to reconcile the research goals of identifying HIV infection, with the need to engage in intensive risk-reduc-

tion counseling. The focus on prevention needs to be very clear to avoid confusion. Communities also need to be sensitized to the fact that, no matter how intensive risk-reduction counseling is, some individuals will remain at risk for HIV infection.

Comprehensive risk-reduction counseling needs to include an assessment of individual risk and a personalized risk-reduction plan, in conjunction with providing technical (condom use), social (safer sex communication and communication with partners), and interpersonal problem-solving skills<sup>12</sup>. Counselors need to be trained adequately in the provision of this counseling and ensure that its impact is sustained throughout the trial. Improved rates of condom use were observed in CAPRISA cohorts within the first six months of enrollment, but they were often not sustained beyond nine months or a year. Strategies to sustain behavioral changes are therefore greatly needed.

It is also clear that many of these risk-reduction efforts may be even more difficult to implement in the lives of the women we recruit. This is reinforced by data on STIs and pregnancy from the CAPRISA 002 study. Overall, 29% and 19% of women were infected with an STI at baseline and at the first six-month visit respectively, in spite of condom provision and monthly risk-reduction counseling. While none of the women were pregnant at enrollment, there were 32 pregnancies [incidence of 8.5 per 100 person-years (95% CI; 5.6-11.5)] in the cohort. One-third of these pregnancies resulted in early terminations, suggesting a high rate of unplanned or unwanted pregnancy. Pregnancy is one of the key challenges in HIV prevention trials and is one of the main reasons women discontinue participation in a trial<sup>12</sup>. It is becoming necessary to consider requiring hormonal contraception as an inclusion criteria in prevention trials to prevent pregnancy because of inconsistent and unreliable condom use by participants.

Tied to the provision of risk-reduction counseling is the need to provide prompt diagnosis and treatment of STIs—especially those linked to HIV acquisition and transmission. The identification of these infections is also essential for understanding their impact on infection rates in prevention trials.

Male circumcision has been shown to be up to 60% protective for men in terms of HIV acquisition<sup>13-15</sup>, leading the World Health Organization (WHO) and Joint United Nations Programme on HIV/AIDS (UNAIDS) to recommend that male circumcision be promoted as part of an effective HIV prevention program. Opinions presented at the 2007 International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention in Sydney, Australia, indicated that circumcision can also reduce the incidence of other reproductive health problems, such as urinary tract infections in male infants, and penile cancer, chancroid, and human papillomavirus (HPV) in adult men<sup>16</sup>. There is some indication that female partners of circumcised men may also benefit, with lower rates of cervical cancer and Chlamydia<sup>16</sup>. Although there have been strong calls from the WHO and many delegates at the recent South African AIDS conference in Durban, the South African National Health Department has not made clear declarations around universal access to circumcision in South Africa. We believe that prevention studies conducted here in the future will need to ensure that men have access to this relatively inexpensive, proven intervention for reducing the risk of HIV infection. However, this needs to be part

of a comprehensive HIV prevention strategy that includes counseling and testing, treatment of STIs, as well as the provision of male and female condoms.

### Future challenges

One of the other challenges of conducting HIV prevention research is the lengthy ethical review process. All documentation for HIV prevention trials must be reviewed by local ethics committees or Institutional Review Boards (IRBs). South Africa is fortunate to have high quality IRBs attached to the local universities, which provide excellent review capacity and turn-around times. Vaccine trials require further approval from the Institutional Biosafety Committees (IBCs), and capacity in this regard may require further development. In places where IBCs do not exist, they will need to be set up prior to the review of vaccine trial protocols.

Although the expertise of the regulatory bodies to review Phase I and II clinical trials has been developed to a limited extent, further improvement is necessary. Before more Phase IIb test-of-concept trials begin, regulatory bodies need to be prepared for these reviews. Efficacy trials involve new challenges and raise questions about the standard of care available within the general population and offering trial participants the best care available.

Prevention trials also face another challenge of co-enrollment of participants who do not disclose their participation in other prevention trials. In some South African populations, unemployment is very high and participants can take advantage of the monetary reimbursement they receive for time and transportation during a clinical trial. This obviously can have a negative impact with regard to safety of the participants, as well as the validity of data collected in these studies. In addressing this issue, researchers might need to share participant information to exclude co-enrollment, which in turn has confidentiality and other ethical implications. Systems will need to be set up to address this issue. There has been very little, and only anecdotal, evidence of this at CAPRISA trial sites. Researchers are currently working with other investigators at prevention trial sites in and around Durban to avoid this problem in the future.

Lack of infrastructure and human resources is another one of the major obstacles to conducting HIV prevention research in developing countries. Investment into developing human expertise is critical in these settings if we are to meet the laboratory demands of vaccine and microbicide research. Attaining and maintaining trained and qualified laboratory staff on processing peripheral blood mononuclear cells (PBMCs) caused a major delay in all five South African vaccine trial sites for the HVTN 503 (Phambili) trial. And now that immunizations have been stopped, retaining these trained laboratory workers has become another challenge.

It is very difficult in South Africa to attract and retain well-trained and experienced staff in contractual research posts. Undergraduate programs that expose students to research and post-doctoral scholarship funding mechanisms need to be established. Clear career paths for junior researchers must also be designed to ensure retention of staff. Once trained, staff mobility between sites causes frequent disruption and delays in trials.

Notwithstanding the differences and study objectives in the various international research networks, uniformity and standardization of some of the laboratory assays is also essential for

the already overstretched laboratories to run smoothly. Quality control and assurance systems need to maintain stringent standards to ensure quality immunogenicity data from these sites. Immunogenicity assays are currently confined to one or two laboratories in South Africa and when additional Phase IIb and III prevention trials get underway, it will be essential to expand this expertise.

The disappointing results of the STEP study, which led to the premature closure of the Phambili trial, have left the South African HIV vaccine trial investigators with many new challenges, including the key question of how to maintain clinical trial sites. This will impact recently established sites—and those that were solely involved in that trial—the most. The concern about job security among trial staff members must also be addressed. But amidst all these challenges, it is crucial to continue the search for additional, effective HIV prevention strategies. ☺

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# Cooking up candidates

*Safety is key when manufacturing candidate AIDS vaccines for clinical trials, and this involves a costly manufacturing process*

by **Andreas von Bubnoff**

**B**efore every space shuttle launch, all systems and processes are carefully checked and rechecked to prevent anything from going wrong. Failure is unacceptable. The same applies to the manufacture of candidate AIDS vaccines for human clinical trials. "Every step has to be checked and rechecked," says Hildegund Ertl of the Wistar Institute. Each vaccine is unique and during every step of production the candidates must be inspected and adjusted if necessary to ensure they are safe and that they retain their activity.

For a vaccine candidate to be safe, it has to be pure, and the process of eliminating any potentially harmful substances takes substantial time and money. For example, manufacturing an experimental DNA vaccine in a lab takes just a few days, but regulatory agencies like the US Food and Drug Administration (FDA) will not allow vaccine candidates that are made in a research lab to be tested in humans, even for small Phase I clinical trials, says Eddy Sayeed of IAVI. Vaccine candidates for clinical trials are instead manufactured in specialized facilities by individuals with expertise on the production and purification of vaccines. Producing a DNA vaccine in such a manufacturing facility that is safe enough for human trials can take months. Tomas Hanke of the University of Oxford says that just ensuring a DNA vaccine candidate is sterile can take six weeks. "Checking what you have is the expensive and time consuming bit," he adds. Sometimes this means just waiting and watching for contaminating bacteria or fungi to grow.

And since time is money, the price tag to manufacture a vaccine candidate for clinical trials is also several magnitudes higher than making one in a research lab. To make enough DNA vaccine for a Phase I trial in an industrial manufacturing facility costs several hundred thousand dollars, says Hanke, who has his DNA vaccines produced by the UK-based manufacturing company Cobra. In a research lab, the same quantity could be produced for only US\$100. The Vaccine Research Center (VRC) in Bethesda, Maryland, part of the National Institute of Allergy and Infectious Diseases, paid \$12 million to have the company Vical manufacture six different DNA plasmids for the proposed PAVE 100 trial, according to Alan Engbring of Vical. Manufacturing highly-purified viral vector-based candidates is also substantially more expensive than making them in a research lab. A new adenovirus-based AIDS vaccine candidate produced in a research lab for testing in nonhuman primates may cost about \$2,000, Ertl says, but a company that manufactures vaccines suitable for clinical trials charges around half a million dollars.

Much of the manufacturing costs for vaccine candidates used in human trials are due to Good Manufacturing Practice (GMP), a set of standards required by regulatory agencies like the FDA or the European Medicines Agency (EMA) for products that are tested in

humans. GMP conditions require, among other things, that the water used to prepare candidates is injection-grade quality and free of any salts. The air in a GMP-certified facility also needs to be highly purified. In addition, everything a person does is double-checked. "One person does the work and another person watches them and they both sign off and follow the protocols exactly," says Jerald Sadoff, who heads the Aeras Global TB Vaccine Foundation. All measurements are double-checked as well. "When your machine says it's incubating at 37 degrees Celsius," Hanke says, "you have to have a different thermometer inside [to check]." GMP manufacturing also involves following Standard Operating Procedures (SOPs),

which require that every step is carefully developed and reproducible, according to Sayeed. An SOP might detail the steps required to prepare the media that is used to culture viruses.

And keeping up to snuff on GMP isn't cheap: Running a compliant facility costs more than \$100,000 a week, according to Sadoff, whose organization has its own facility to manufacture vaccines against tuberculosis (TB). In fact, 80% of the expense of manufacturing vaccines is from maintaining GMP conditions, estimates Andreas Neubert, head of vaccine production at IDT Biologika GmbH, a German company that manufactures modified vaccinia Ankara (MVA)-based vaccines for IAVI and Oxford University, among others. Ertl, who will have her adenovirus-based AIDS vaccine candidates made by the California-based company SAFC Pharma, agrees. "Use of a [GMP] facility is a big chunk of the price."



**Use of  
a [GMP]  
facility  
is a big  
chunk of  
the price**  
Hildegund Ertl

## **No pathogens, please**

But there is more to vaccine production than GMP. Each type of vaccine—and the cells used to manufacture them—also needs to be free of any pathogens or other potentially harmful substances.

To manufacture DNA vaccines, the plasmids are grown in bacteria whose outer membrane contains endotoxins. These are a concern because they are toxic to humans—they can interact with macrophages that release Tumor Necrosis Factor, a compound that causes the release of nitric oxide, which in turn causes blood vessels to relax. As a result, people go into shock, Sadoff says. To remove endotoxins, DNA vaccines are filtered through substances that bind to them. Tests for remaining endotoxins are done by conducting safety studies in rabbits, which are very sensitive to endotoxins, according to Sadoff.

Some viral vector-based vaccines are grown in cells from chicken eggs, and pathogens found in these eggs, such as avian viruses or bacteria, are also an issue. Sendai virus vectors are grown in cells in the so-called allantoic sac of chicken eggs, and MVA vectors are grown in chicken embryo cells called fibroblasts. The eggs used for such vaccines need to come from chickens held under "specific





*A laboratory worker at the German vaccine manufacturer IDT handles a roller bottle to be filled by a robot with sterile cell cultures containing modified vaccinia Ankara (MVA) vector and growth media.*

**People ask if there is scarcity for large scale HIV vaccine manufacturing. The answer is yes, but when it comes to crunch time, the capacity will be identified**

**Eddy Sayeed**

pathogen-free” conditions. Germany-based MVA vaccine manufacturer IDT buys such eggs at about 20 times the cost of regular eggs, Neubert says. Inactivated flu vaccines are also grown in chicken eggs, but these are much easier to purify because the cells can be treated with the chemical formalin to kill contaminants. This cannot be done for live viruses like MVA because such purification would also inactivate the vector.

Vaccines that use adenoviruses as a vector are typically grown in human cell lines, which also need to fulfill certain safety criteria before getting approved by regulatory agencies such as the FDA, Ertl says. For one thing, the host cell lines need to be free of prions, infectious protein particles that are believed to cause diseases in animals, such as mad cow disease, and a fatal variant, Creutzfeldt-Jakob disease, in humans. To ensure the cell lines used to grow adenovirus vector-based vaccine candidates are free from prions, their entire history must be documented. That history has to show that the cells have always been grown in serum from uninfected animals. “You can’t even have a one- or two-week blank in that record,” Sadoff says. The cell lines and the viruses grown in them also need to be carefully inspected for many other contaminating

viruses and pathogens, Sayeed says. Cancer is another concern, since a vaccine candidate contaminated with DNA from human cells might cause tumors. To avoid this, the FDA requires the removal of most of the host cell DNA. Together, these strict requirements are the reason that only a handful of cell lines are available for manufacturing adenovirus-based vaccines.

#### **Keeping it consistent**

Consistency is another challenge for vaccine manufacturers. “It’s unethical to do a trial with something that would never be reproducible,” Sadoff says. Ensuring reproducibility requires a series of evaluations. For recombinant proteins, these evaluations include checking their molecular weight, whether the vaccine candidate reacts with the right antibodies, and how potent an immune response the candidate induces. “These potency assays are the most difficult because they have the highest variability and have to be very well characterized,” Sadoff says.

The concern that vaccines may change during production is indeed justified. For example, replication-defective adenoviruses grown in certain cell lines, such as HEK 293, can regain the ability to replicate. This poses safety concerns. Typically, adenoviruses used

as vaccine vectors are engineered so they lack certain genes necessary for replication, such as E1. This gene is transferred instead into the cell line used to grow the virus. As a result, the cell line is able to divide indefinitely, making it easier to culture the cells. But sometimes the gene is transferred from the genome of the host cells into the virus genome because of similarities between the DNA sequences of the host cells and the adenovirus. If this transfer occurs, the adenovirus can once again replicate. "If you have too many of such [replicating] viruses, you have to throw away the whole batch," Hanke says. Other cell lines used to grow adenovirus, such as PER.C6 and HER 96, don't have this problem because their genomes are not as similar to the adenovirus genome, according to Sayeed.

Viral vectors grown in cell lines can even eject the HIV genes they carry. "They kick the transgene out," Sadoff says. "Almost all the MVAs that have been tested have had this problem." One reason is that some HIV immunogens, such as the Env protein, can be toxic to cells and make the viral vector genetically unstable, Sayeed says. Manufacturers therefore have to repeatedly test the vector to verify the HIV inserts are still there.

#### **Developing the process**

Along with safety and consistency, the production process also needs to be optimized before larger quantities of vaccine candidates are made. With DNA vaccines, manufacturers must find the most efficient host bacteria and the ideal time to stop bacterial growth before harvesting the DNA. Manufacturers also have to check the ideal growth conditions of cultured cells. Some chicken embryo fibroblasts that are used to grow MVA prefer growing adherent on surfaces, while others grow in suspension, according to Neubert. And growth efficiency drops as soon as HIV transgenes are introduced into the vector, Sayeed says. In the case of MVA, just inserting a single HIV gene renders it 10 times less productive.

Once vaccine production is optimized on a large scale, the price is likely to drop. Making large batches is easier for some vaccines than others, depending on the process. It is relatively easy to make large batches of DNA vaccines that are manufactured in bacteria and large-scale manufacturing could bring the per-dose price of a DNA vaccine down from \$1,000, to around \$4, Sayeed says. Vaccines that use adenovirus as a vector can also be made rather easily on a large scale because the human cell lines they are grown in can divide indefinitely.

However, scaling up becomes more difficult for MVA-based vaccines. Since the chicken embryo cells they are grown in do not multiply indefinitely, the virus always needs to be harvested from fresh eggs. As a result, manufacturing an MVA-based vaccine for millions of people could require 100,000 eggs per week, Sayeed says. Companies are now developing new avian cell lines for large-scale production to circumvent the dependency on fresh eggs. Another disadvantage of manufacturing MVA-based vaccines is that a rather

large volume of vaccine culture is needed to get a small amount of vaccine, according to Sadoff, because it is less concentrated.

#### **Got GMP?**

Finding a manufacturer that can make a vaccine under GMP conditions is not that easy, says Sayeed, who is in charge of finding companies to manufacture vaccines IAVI has developed with its partners. That's especially true for vaccines based on viral vectors. "There is a waiting list," Sayeed says. Only a handful of companies worldwide can do the work, he adds, and some of them are booked for at least nine months.


A few years ago, the manufacturing team at IAVI did a survey and found that of about 100 companies, only a handful could really manufacture vaccines, among them Transgene in France, Cobra in the UK, SAFC Pharma and Vical in the US, IDT in Germany, and


Henogen in Belgium. Many companies that used to be involved in manufacturing vaccines have abandoned the business because it's not profitable enough, Sayeed says. There are also contract manufacturers in countries like India, South Korea, Brazil, and China that can do the job, but researchers are hesitant to go there because they are concerned about protecting intellectual property, according to Sayeed.

Meanwhile, some academic and non-profit organizations have started to make candidate vaccines in their own facilities. The VRC, for example, has its own facility and the University of Oxford may also use its own facility to manufacture adenovirus-based AIDS vaccine candidates in the future. This is generally cheaper than using a commercial manufacturer, according to Pru Bird, head of research at the Oxford facility. AERAS also manufactures


vaccines in its own facility, and last year, the Canadian government, in collaboration with the Bill & Melinda Gates Foundation, announced the creation of the Canadian HIV Vaccine Initiative (CHVI). The initiative has proposed building a vaccine manufacturing facility in Canada, according to Ingrid Wellmeier of the Public Health Agency of Canada, in response to a limited global capacity.

There are currently no dedicated large-scale facilities in place that could immediately take over production if an AIDS vaccine was proven to work in efficacy trials, Sayeed says. Manufacturers have to strike a careful balance between building a facility—which can take several years and cost a significant amount—and the risk that it may become useless if a vaccine eventually fails in late stage clinical trials. One strategy adopted by some of the big pharmaceutical companies, with several products in the pipeline, is to build generic facilities that can accommodate different types of vaccine technologies. This way, their construction is flexible enough to switch to the vaccine that is successful, even midway into the building process.

Sayeed, for his part, remains optimistic. "People ask if there is scarcity for large-scale HIV vaccine manufacturing. The answer is yes, but when it comes to crunch time, the capacity will be identified." 



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**Jerald Sadoff**

## Trial results presented at CROI

Results from two recently-conducted clinical trials of different prime-boost AIDS vaccine regimens were presented at the 15th Conference on Retroviruses and Opportunistic Infections (CROI) held February 3-6 in Boston. The first trial, conducted by the HIV Vaccine Trials Network (HVTN) at multiple sites in the US, tested two vaccine candidates developed at the Emory Vaccine Center and now licensed to the biotechnology company GeoVax. The prime-boost immunization regimen consisted of a DNA candidate and a modified vaccinia Ankara (MVA) vector-based candidate, both expressing HIV Gag, Pol, and Env proteins.

This trial, known as HVTN 065, evaluated the safety and immunogenicity of two doses of the candidates—10 volunteers received two injections of a low dose of the candidates (0.3 mg DNA followed by a 10,000,000 viral particle dose of the MVA vector-based candidate). Another 30 volunteers received two injections of the candidates at a high dose (3 mg DNA followed by a 100,000,000 viral particle dose of MVA). Immune responses were measured two weeks following each MVA boost using ELISPOT assays to measure secretion of interferon (IFN)- $\gamma$  and interleukin (IL)-2 by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

After the second MVA injection at the lower dose, 87% of participants were considered responders based on their CD4<sup>+</sup> T-cell responses to any of the HIV immunogens included in the candidates. After the second MVA injection at the higher dose, 77% of participants mounted CD4<sup>+</sup> T-cell responses to any of the included HIV immunogens. Overall the CD8<sup>+</sup> T-cell responses were much lower. At the higher dose, 42% of participants were considered responders, compared to 33% in the low-dose group. Harriet Robinson, who recently announced she will be leaving Emory University to join GeoVax full time, presented the immunogenicity data at CROI, saying the vaccine candidates exhibited “excellent cytokine production profiles.”

The Gag-specific responses were boosted by the second administration of MVA and Robinson said this was promising since immune responses to HIV Gag correlate with successful control of HIV in infected individuals. Bruce Walker of

Massachusetts General Hospital, who was in the audience, pointed out that based on his work, it is actually the breadth of Gag-specific responses that seems to be important in control of HIV infection. Robinson said data on the breadth of Gag-specific immune responses was not yet available from this trial.

Based on these results, the high-dose regimen will be tested in the second part of this trial, involving two groups of 30 volunteers. One group will receive a single injection of the DNA candidate and two MVA boosts, the other will receive three injections of the MVA-based candidate.

Immunogenicity data from a Phase I/II trial in Mbeya, Tanzania of the DNA and adenovirus serotype 5 (Ad5)-based candidates developed by the Vaccine Research Center (VRC), part of the US National Institute of Allergy and Infectious Diseases, were also presented at CROI. This trial was conducted by the United States Military HIV Research Program (USMHRP) and was one of a series of Phase I and II studies with the VRC's candidates in preparation for the originally-planned Phase IIb test-of-concept trial known as PAVE 100.

The DNA plasmid vaccine candidate encodes Gag, Pol, and Nef from HIV clade B and Env from HIV clades A, B, and C. The Ad5-based candidate encodes the same immunogens except Nef. In this trial, 60 volunteers received three injections of the DNA vaccine candidate followed by a single booster immunization with the Ad5 candidate, or placebo. Immune responses in 40 of the volunteers were measured using IFN- $\gamma$  ELISPOT assays. After the Ad5 boost, 80% of vaccinees had at least 55 spot-forming cells per million peripheral blood mononuclear cells (PBMCs). The majority of immune responses were directed toward Env, but researchers were not able to measure the magnitude of Gag-specific IFN- $\gamma$  responses. This analysis will be completed later with frozen cells. The majority of participants in this trial had high levels of anti-Ad5 antibody, yet all individuals mounted some level of HIV-specific immune responses following receipt of the Ad5 candidate, indicating that pre-existing immunity to the vector from exposure to the naturally-circulating virus did not completely mitigate the effect of the Ad5 boost.

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## Circumcision progress?

Last year, the US-based news magazine *Time* ranked male circumcision as the number one medical breakthrough of 2007 because of its potential to slow the spread of HIV. Results from three randomized, controlled clinical trials have shown that circumcision cuts the risk of HIV infection in men by roughly 60%. Subsequently, the World Health Organization (WHO) issued guidelines urging countries to consider adding male circumcision to their existing HIV/AIDS prevention strategies, but to date, only a handful of health ministries in sub-Saharan Africa—the region most severely affected by HIV/AIDS—have started developing national policies on circumcision. This has spurred some public health officials to question the delay.

In an editorial published in the January issue of the journal *Future HIV Therapy*, Daniel Halperin, senior research scientist at Harvard University, and colleagues emphasized the benefits of male circumcision and called upon countries, international leaders, and donor agencies to introduce safe circumcision practices in sub-Saharan Africa. Halperin says that so far approximately nine African governments have conducted consultations with the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the WHO. “I think in every case, after doing the consultation, they decided that they wanted to have a circumcision program or policy,” he says. But so far few policies have been established. “A lot of these countries are on their way, but only Kenya and Rwanda have actual policies as far as I know.”

Kenya’s Ministry of Health published its official policy on male circumcision in September 2007. The Kenyan policy stipulates that safe, voluntary male circumcision should be promoted in conjunction with other HIV prevention strategies, but provides no indication of when circumcision programs will be implemented.

At the 15th Conference on Retroviruses and Opportunistic Infections (CROD), held February 3-6 in Boston, Bertran Auvert, an investigator on the first trial of adult male circumcision in South Africa, provided an update on national circumcision policies in Africa. “Today, no country has started the rollout of male circumcision,” Auvert said in a conference session. He cautioned that even once policies are adopted, implementation will take time.

Previous surveys of men in sub-Saharan Africa found the

procedure would be highly acceptable—between 65% and 81% of men said they would consider undergoing circumcision if it provided some protection against sexually-transmitted infections. Yet Auvert argues that this is not an indication that men will voluntarily opt to be circumcised. “We need to say: ‘Now, safe and free male circumcision is available. Will you go? And when?’” Studies are ongoing to address this question and results should be available within a year.

There are also many other challenges that have contributed to delays in instituting male circumcision programs, including cultural hurdles, a shortage of trained professionals, and financial constraints. While the US President’s Emergency Plan for AIDS Relief (PEPFAR) has agreed to fund circumcision programs, the governments and health ministries need to specifically request this support. “Once they ask for it, it’s like anything else, it takes a while for the money to come down,” says Halperin. “It’s going to vary in different places but I’m sure there will be a lag before things really get going.”

Study findings released at CROI indicate that while male circumcision provides significant protection against HIV transmission in men, it offers no direct benefit to the female partners of HIV-infected circumcised men (see *Clues from CROI*, page 1). Results of this study, conducted in Uganda, also suggest that women who engage in sexual activity with a recently circumcised HIV-infected partner before his wound has fully healed actually increase their risk of acquiring HIV.

Yet providing circumcision services to adult men in areas with high HIV prevalence could considerably reduce the number of new infections. Computer modeling studies conducted by the WHO to determine the potential impact of circumcision on the course of the HIV epidemic suggest that if all males in sub-Saharan Africa were circumcised, two million HIV infections could be averted over the next 10 years. An additional 3.7 million new infections could be prevented over the following decade.

Evidence for the potential impact of circumcision programs can already be seen on the population level, says Halperin. For example, in Cameroon, a country where male circumcision is common practice, the adult HIV prevalence rate is only 5%, whereas in Botswana and Swaziland, countries where the majority of men are uncircumcised, adult HIV prevalence rates are up to five times higher. “It’s not just about modeling. We can actually see the real-world impact,” says Halperin.