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Review

Novel Approaches to Vaginal Delivery and Safety of Microbicides: Biopharmaceuticals, Nanoparticles, and Vaccines

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ABSTRACT

The HIV-1 epidemic remains unchecked despite existing technology; vaccines and microbicides in development may help reverse the epidemic. Reverse transcriptase inhibitors (RTIs) formulated in gels tenofovir (TFV) and IVRs (dapivirine) are under clinical development. While TFV or similar products may prove successful for HIV-1, alternatives to RTIs may provide additional benefits, e.g., broader STI prevention. Biopharmaceutical agents under development as microbicides include cyanovirin, RANTES analogues, commensals, and Mabs. Cost of manufacturing biopharmaceuticals has been reduced and they can be formulated into tablets, films, and IVRs for vaginal delivery. Nanotechnology offers a novel approach to formulate microbicides potentially leading to uniform epithelial delivery. Delivery through vaginal mucus may be possible by controlling nanoparticle size and surface characteristics. Combining prevention modalities may be the most effective means of preventing STI transmission, importantly, co-delivery of microbicides and vaccines has demonstrated. Finally, the safety of microbicide preparations and excipients commonly used can be assessed using a mouse/HSV-2 susceptibility model. Screening of new microbicide candidates and formulation excipients may avoid past issues of enhancing HIV-1 transmission. This article forms part of a special supplement covering several presentations on novel microbicide formulations from the symposium on "Recent Trends in Microbicide Formulations" held on 25 and 26 January 2010, Arlington, VA.

Contents

Abbreviations: RTIs, reverse transcriptase inhibitors; IVRs, intravaginal rings; HIV-1, human immunodeficiency virus-1; IUD, intrauterine device; STI, sexually transmitted infection; SRH, sexual and areproductive health; MPT, multipurpose technology; ARV, antiretroviral therapy; Mabs, human monoclonal antibodies; EVAc, poly (ethylene-co-vinyl acetate) ;PLGApoly(lactide-co-glycolic acid); PSA, poly(sebacic acid), poly(lactic acid); MPPs, mucus-penetrating nanoparticles; PEG, polyethylene glycol; FITC, fluorescein isothiocyanate; N9, nonoxynol-9; HSV-2, human simplex virus type 2; CHX, chlorhexidine; ID_{50}, 50% inhibitory dose; APIs, active pharmaceutical ingredients; SDS, sodium docecylsulfate; EDTA, ethylenediaminetetraacetic acid; GML, glycerol monolaurate; PrEP, pre-exposure prophylaxis ;RTIsreproductive tract infections; TFV, tenofovir ;HEChydroxethylcellulose; NHP, non-human primate.

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1. Introduction

Prevention of HIV-1/STI transmission initially focused on evaluation of non-specific entry inhibitors such as cellulose sulfate and Carraguard. Second generation microbicides containing RTIsare currently the predominate products in clinical development. These agents are formulated in vaginal gels and IVRs; alternatives include fast dissolve tablets and films. All these formulation approaches are based on technology used in commercial vaginal products. However recent advances in therapeutic approaches to HIV-1 treatment and prevention along with advances in drug delivery technology permit new formulation approaches to prevent transmission of HIV-1 and other STIs.

This review covers vaginal delivery of biopharmaceuticals (e.g., peptides, antibodies, and immunogens), nanoparticles,and combinations of vaccines and microbicides. Biotechnology products typically require sophisticated formulations to deliver these agents over the optimal time course and in a bioactive form. Examples of the use of nanoparticles are reviewed with an emphasis on their potential ability to penetrate vaginal mucus and deliver protective agents. The formulation and delivery of combinations of low molecular weight microbicides with biopharmaceuticals, e.g., coadminstration of RTIs and vaccines against HIV-1 appears to be feasible.

Safety evaluation of new microbicide formulations for vaginal and rectal use is of significant importance sinceboth APIs and excipients may potentially increase susceptibility to HIV-1 transmission. A model is described herein that may be useful in screening various candidate microbicide formulationsfor increased susceptibility to viral infection.

This paper forms part of a group of seven reviews covering presentations from the Trends in Microbicide Formulations Workshop held 25 and 26 January 2010 in Arlington, VA, USA. Current understand of HIV-1 transmission and the role of microbicides (Hladik and Doncel, 2010), preclinical evaluation of microbicides (Doncel and Clark, 2010), gel, film, and tablet formulations (Garg et al., 2010), intravaginal rings (Malcolm et al., 2010), clinical evaluation of microbicides (Morrow and Hendrix, 2010), and dual protection (Friend and Doncel, 2010) comprise the other reviews.

2. Unsafe sex

Unsafe sex is the second most important risk factor for disability and death in the world's poorest communities and the ninth most important in developed countries (Glasier, 2007). Every year, more than 120 million couples have an unmet need for contraception (80 million women have an unintended pregnancy, half a million die from complications associated with pregnancy, childbirth, and the postpartum period), and 340 million acquire a STI. Marketing studies in the US (Holt et al., 2006) and acceptability studies internationally (Simons-Rudolph et al., 2008) suggest the importance to end-users of combining STI and pregnancy prevention. Although there are products for preventing pregnancy (e.g. the pill, IUDs, diaphragms) and STIs (condoms),the epidemic incidence rates of both unintended pregnancy and STIs clearly illustrate the need for MPTs with improved acceptability (Friend and Doncel, 2010).

Although the first microbicides to enter clinical trials have activity under certain conditions (in vitro, explants, and animal models) against sperm and a broad array of STI pathogens (see Table 1; Whaley, 2004; McGowan, 2006), second generation microbicides are focused on prevention of HIV-1 transmission (Table 1).

Researchers, policymakers, product developers, and donors have begun to address multiple SRH challenges by advocating for commitment to a new generation of technologies that simultaneously address multiple risks and needs – technologies that will alleviate the heavy health and economic toll that STIs, unintended pregnancy, and other reproductive tract infections impose on women, their families, and their communities. Creating MPTs for SRH will help the global community accelerate progress toward internationally agreed-upon health and development targets, including the Millennium Development Goals (MDGs) and the Global Health Initiative (Cates, 2010). Achieving this vital goal will require a determined, well-coordinated and innovative effort.

3. Noncoital delivery

Data from published microbicide trials make it clear that microbicides which must be used in close association with intercourse (coital methods) are challenging to use consistently. In the Carraguard trial (Johansson, 2008), an objective measure of gel use indicated that study gel was used in only 43% of intercourses. More problematic for demonstrating the efficacy of coital methods, is the low frequency of "informative coital acts", i.e., acts where the study gel was used and a condom was not used, the only acts that can assess microbicide effectiveness. Due to condom promotion in trials (a method of known effectiveness promoted to all trial participants), and due to the limited consistency of use of coitally-dependent microbicides, the fraction of informative acts

Table 1
Activity and mechanism for microbicides in development (adapted from Zeitlin et al., 2009).

	Narrow Spectrum	Broad Spectrum
Non-Specific Mechanism		1st Generation BufferGel Carraguard PRO 2000 VivaGel Cellulose Sulfate* Savvy* Nonoxynol 9*
Specific Mechanism	2nd Generation Tenofovir Dapivirine UC781	3rd Generation Combinations OR strategies with versatile manufacturing platforms (e.g. antibodies, commensals)

* possible harm observed in clinical trials.

in trials can be low. In one published microbicide trial (Feldblum et al., 2008), the percentage of sex acts in which the study gel was used and a condom was not was only 9%, severely limiting study power. Thus, the effectiveness of coitally-dependent microbicides is reduced by non-adherence and the ability to demonstrate effectiveness in clinical trials can be severely compromised.

4. Industrialization: Translating recombinant biopharmaceuticals into microbicides

Biopharmaceuticals are proteins (lectins, antibodies, defensins, subunit immunogens), nucleic acids (DNA, RNAi, or antisense oligonucleotides), and viruses or bacteria used for prevention or therapy. Although biopharmaceuticals have been available for several decades, use of these agents has been limited in some global health products, such as microbicides, due to high cost and constrained production capacity.

The US Food and Drug Administration identified the industrialization process as being rate-limiting for development of new technologies (Critical Path, FDA, 2004). The manufacturing challenges involved in successful industrialization are complex, and highly underrated in the scientific and funding community. Problems in physical design, characterization, manufacturing scale-up, formulation, packaging, and quality control routinely derail or delay development programs.

5. Solutions to formulation of biopharmaceutical microbicides

Biopharmaceuticals have the specificity and versatility for the development of MPTs and have the potential for *non-coital, sustained delivery*. Advances in manufacturing in bacteria and yeast (RANTES), bioengineered commensals (cyanovirin, RANTES, two-domain CD4), and plants (antibodies, griffithsin, cyanovirin), may solve cost and capacity constraints for biopharmaceuticals. The increased availability of APIs derived from biotechnology is now facilitating the development of formulated biopharmaceuticals for sustained delivery. Although other biopharmaceuticals are in development as microbicides (e.g., siRNA; see Palliser et al., 2006), the first part of the review focuses onmanufacturing formulation and delivery of protein microbicides currently in advanced preclinical or clinical development.

5.1. Recombinant RANTES

Topically applied recombinant chemokine analogues fully protect macaques from vaginal simian-human immunodeficiency virus challenge (Veazey et al., 2009). However, chemically synthesized chemokine analogues present cost challenges.

5.1.1. Manufacturing

Recombinant anti-HIV chemokines (5P12-RANTES, 6P4-RANTES) can be produced with biotechnology (Gaertner et al., 2008). Recombinant 5P12-RANTES has been manufactured utilizing bacterial and yeast fermentation (Oliver Hartley, personal communication). The biopharmaceutical manufacturing costs are expected to be markedly reduced compared with chemical synthesis, and production capacity greater.

5.1.2. Formulation

Recombinant 5P12-RANTES has shown stability at 55 °C for over 30 days in HEC gel (Oliver Hartley, personal communication). In addition, 5P12-RANTES and 6P4-RANTES have shown promising stability at elevated temperatures and low pH, and after incubation with human cervicovaginal lavage samples (Cerini et al., 2008).

Since HEC gel is widely used and considered safe (Tien et al., 2005), 5P12-RANTES could be combined with other agents to produce a multipurpose microbicide. Sustained activity may be feasible if 5P12 RANTES is incorporated into devices such as IVRs.

5.2. Bioengineered commensals

Bioengineering of H_2O_2-producing vaginal Lactobacillus as a platform technology for mucosal delivery of protein-based microbicides (RANTES, Cyanovirin-N, two-domain CD4) presents a promising approach for the re-establishment of beneficial flora and development of cost-effective female controlled preventatives (Secchi et al., 2009).

5.2.1. Manufacturing

The production of a properly formulated live microbicide product for mucosal delivery is a major challenge, but significantly improved cell viability can be achieve by optimizing the bacterial fermentation and preservation process (Liu et al., 2010). Lactobacillus has been fermented up to 10 billion CFU/ml with greater than 90% viability using medium free of animal sources, an anti-foaming agent, pH control with ammonium hydroxide, and glucose supplementation.

5.2.2. Drying and preservation

To preserve Lactobacillus during lyophilization or freeze drying, addition of sodium ascorbate in combination with another antioxidant and polyol into the preservation matrix (e.g., skim milk and trehalose) significantly increases cell viability of dried Lactobacillus powders. Since pH affects cell recovery of the dried cells during the rehydration process, a buffered preservation matrix was developed to produce a highly viable product at 100 billion CFU/gram.

5.2.3. Formulation

Dried Lactobacillus can be incorporated into vaginal tablets or dispersed as a powder from a vaginal applicator. Cyanovirin secreting Lactobacillus has been incorporated into a film formulation (personal communication, L. Rohan).

5.2.4. Sustained delivery

The success of live microbial microbicides will depend, in part, on the level and duration of in situ Lactobacillus; a Chinese rhesus macaque model for vaginal Lactobacilllus colonization and live microbicide development has been established (Yu et al., 2009); prolonged colonization of a human vaginal *Lactobacillus jensenii* in macaques has been demonstrated in this model.

5.3. Recombinant antibodies

Because of their high potency, specificity, and safety profile, Mabs are leading candidates for multipurpose microbicides. Mabs are uniquely capable of being specific and broad spectrum, i.e. multi-antibody prevention products can have diversity in mechanism (neutralization, agglutination, trapping in mucus) and target. A microbicide containing three HIV antibodies (MabGel) that were manufactured in mammalian cells is currently in early phase clinical trials (Morris et al., 2010).

5.3.1. Manufacturing

The ability to produce biopharmaceuticals "on demand" in large quantities, at low cost is a feature of deconstructed viral vectors that achieve rapid, high-level expression of Mabs in *N. benthamiana* (Giritch et al., 2006). Other viral vectors, e.g., Gemini viruses (Huang et al., 2010), provide comparable levels of expression. The technology is in essence an en masse infiltration of whole mature plants with a diluted Agrobacterium suspension carrying T-DNAs encoding viral replicons. The result is a high copy number of RNA molecules that encode the desired biopharmaceutical, reducing the time needed to produce a protein to 4–10 days. The technique appears to be the most rapid route from genes to full-length, assembled Mab (Hiatt and Pauly, 2006) and immunogens. Moreover, increasing the volume of Mab-containing biomass does not require changes in growing conditions, infection procedures, or equipment, and is directly scalable. Unlike transgeneic plant expression systems, no genes are incorporated into the plant genome, and as a result, there is no risk of propagation of the transgene from pollen, seeds, or other routes. Further, no intact and replication-competent virus is produced, eliminating risk of virus mediated spreading of the recombinant genes. Finally, the entire production system is performed indoors in enclosed growth rooms, providing an additional layer of quality control and security.

5.3.2. Low cost, large capacity spray drying

Incorporation of biopharmaceuticals into thin films, tablets or IVRs requires a stabilized powder precursor, which is simple to manufacture by spray drying. Spray drying has seen increasing utilization as a process to stabilize proteins by rapid vitrification in the presence of amorphous sugars. In addition to its ability to control powder properties (e.g. particle size, particle morphology, powder density and surface composition), the key advantages of spray drying are: shorter process cycle time (i.e., more batches per unit time), scalability (i.e., large batch size per unit operation, requiring less number of production units), and the ability to process at atmospheric pressure. An advantage to spray drying over conventional lyophilization is that powders of appropriate size range can be generated and pressed into vaginal tablets or incorporated into vaginal films.

GMP manufactured HSVgD and CCR5 Mabs that are spray dried yield less than 0.5% loss in monomer content under harsh processing conditions (T in/T out, 90/70 °C). The spray dried Mabs are stable at 37C for more than three months, and could be incorporated into film, tablets or vaginal rings.

5.3.3. Sustained delivery of antibodies from vaginal devices

Antibody-releasing vaginal rings made of poly (ethylene-co-vinyl acetate [EVAc]; Radomsky et al., 1992; Sherwood et al., 1996) have been shown to prevent HSV transmission in mice. EVAc is biocompatible and has FDA approval for use in the human uterus (Progestasert®) and cervicovaginal lumen (NuvaRing®). Since human cervical mucus does not act as a barrier to antibody diffusion (Saltzman et al., 1995), antibodies released from EVAc vaginal rings may be able to distribute throughout the vaginal lumen and provide protection to the entire vaginal epithelium. The target is for a one week to one month EVAc ring releasing MAbs. To simplify manufacturing and enable reuse of rings, sustained release matrix (HPMC) tablets could be manually added by users on a weekly basis to rings that have cavities for tablets; such devices may be relatively low in cost.

Developers of biopharmaceutical microbicides are addressing the challenges of industrialization and sustained delivery, ensuring that biopharmaceuticals are viable candidates for the new generation of multipurpose microbicides.

6. Mucus-penetrating nanoparticles (MPPs) for sustained microbicide delivery

Nanoparticle technology offers potential for developing sustained release 'once-a-day' vaginal application of microbicides that may improve user-acceptability by being independent of coitus. Of potentially equal importance, mucus-penetrating nanoparticles may provide advantageous distribution (deployment) of microbicides throughout the reproductive tract, including the highly folded epithelial surfaces of the vagina. Moreover, sustained-release from nanoparticles could reduce transient peaks in drug concentration inherent with gel delivery and avoid high local concentrations that occur in the proximity of sustained release devices such as IVRs.

A variety of nanoparticle systems can provide sustained drug delivery for greater than 1 day, especially for small hydrophobic molecules like many of the ARVs underdevelopment. For example, nanoparticles can be made with a variety of biodegradable and biocompatible polymers, such as PLGA, PSA, and PLA, and these can release a wide range of low-molecular weight drugs. However, these conventional nanoparticles are mucoadhesive (Tang et al., 2009), a property that was originally thought to be beneficial for mucosal drug delivery. Mucus is secreted and shed continuously, so mucoadhesive particles lodged in luminal mucus may be shed rapidly (Cone, 2009; Lai et al., 2009). Relatively few particles would be expected to reach the unstirred layer of mucus below the superficial mucus adherent to vaginal surfaces, and even fewer actually reach the epithelial surface.

Thus, mucus represents a *barrier* for mucosal drug delivery using conventional nanoparticles (see the special issue of Advanced Drug Delivery Reviews, February 2009, that addresses this problem). Recently, by mimicking the mucus-penetrating characteristics of viral pathogens, Lai et al. (2007) developed MPPs capable of diffusing through undiluted human cervico-vaginal mucus in vitro almost as rapidly as water. Methods for optimizing non-mucoadhesive surface-coatings for MPPsare being developed (Tang et al., 2009; Wang et al., 2008). MPPs must be densely coated with neutral hydrophilic surfaces to prevent mucin fibers from forming hydrophobic bonds with the particle. Hydrophilic surfaces can be created using low molecular weight PEG (Wang et al., 2008).

MPPs have been used to assess pore-size, or mesh-spacing in fresh minimally diluted or perturbed samples of human

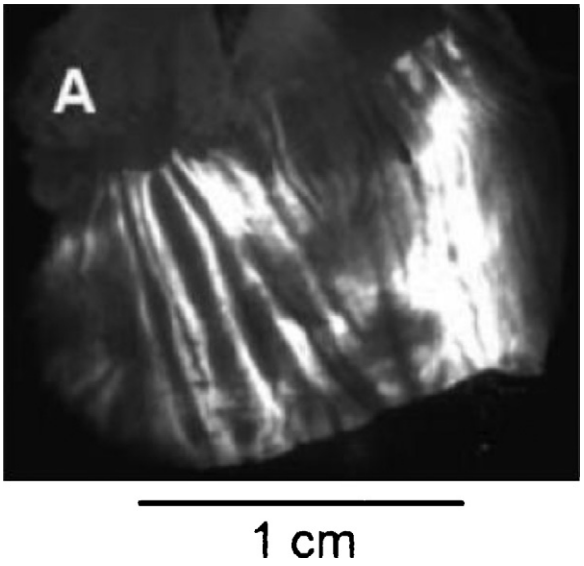

Fig. 1. Epifluorescent image of entire mouse vaginal epithelial surface (Cone et al., 2008).

cervicovaginal mucus; the average pore size is 340 ± 70 nm (Lai et al., 2010). HIV-1 and many other viral pathogens are significantly smaller than this pore size and, hence, these virusestheoretically diffuse through mucus to reach their target cells as long as the viral surface is non-mucoadhesive. Interestingly, HIV-1 is mucoadhesive when exposed to human cervicovaginal mucus natively acidified with lactic acid to pH ~4. When this mucus is neutralized, as occurs when the ejaculate is present, HIV-1 diffuses through mucus (Lai et al., 2009).

The ability of pathogens to penetrate the mucus barrier suggests that, to be highly protective, microbicides must also penetrate the mucus barrier. MPPsmay be able to perform this function, especially when delivering hydrophobic drugs that, if not encapsulated, may partition into the first epithelial surface they reach. Such drugs may fail to diffuse deeply into vaginal folds (rugae) because they may enter and be retained on the outermost luminal surfaces of the epithelium. This potential problem was first recognized with detergent-based microbicides: detergent micelles have hydrophilic surfaces and readily diffuse through mucus, but the detergent monomers rapidly partition into the first epithelial surface the micelles contact. Thus, detergents do not uniformly coat the vaginal surface; instead they predominantly enter only those regions that directly face the lumen. This process can be observed by fluorescently labeling cells disrupted by the detergent using a 'dead-cell' dye, YOYO-1, as shown in Fig. 1 (Cone et al., 2006). The vagina is dissected out and flattened between two microscope slides to open the vaginal folds (rugae). The bright stripes are regions of disrupted and stained cells that directly faced the lumen, the dark stripes are infoldings that N9 did not reach. Similar images can be obtained using relatively hydrophobic fluorophores, such as FITC, reveal non-uniform distribution when attempting to deliver hydrophobic drugs to the vaginal surface. In contrast, hydrophilic fluorophores, and MPPs, diffuse through mucus to reach the entire epithelial surface, i.e., and they do not partition into or adhere to, only those surfaces that directly face the lumen. The relevance of these findings requires confirmation in studies involving time frames relevant to microbicide delivery (i.e., longer than 10 min). Also the relationship, if any, between this study (Cone et al., 2006) and nanoparticle penetration needs to be established.

In summary, mucus-penetrating particles may provide sustained microbicide delivery for >24 hoursand deliver hydrophobic microbicides more uniformly to the entire vaginal epithelium than application via gels or IVRs. HIV-1 is natural mucus-penetrating particle, and MPPs are likely to reach every epithelial surface that HIV-1 can reach. MPPs may thus enable development of 'once-a-day' methods that also achieve a higher level of protection by delivering and deploying microbicides to all the surfaces that can be reached by mucus-penetrating pathogens.

7. Vaccine/microbicide combinations

Combining the three major prevention modalities currently in development (vaccines, pre-exposure prophylaxis [PrEP], and microbicides) may be the most effective means of preventing HIV-1 transmission.

Macaque models have shown that 30–60 min of exposure is all that is needed to establish SIV infection. Infected cells can be detected in mucosal tissue within 24 h. Dissemination to the draining lymph nodes occurs within 24–72 h. With vaccines, local immune memory may be inadequate to prevent infection, so regular and repeated intravaginal vaccination may be required to maintain immune function at a level required to prevent infection.

While HIV-1 transmission is not completely understood (Anderson, 2010; Anderson et al., 2010), researchers have made substantial progress in understanding the types of virus that ultimately infect an individual. Eighty percent of HIV-1 infected subjects are infected with a single virion/single quasispecies, while 20% are infected with a few HIV-1 quasispecies. All show strong T cell tropism, which has been validated in Clade B, Clade C, and SIV mucosal transmission. The balance between infection and protection at mucosal surfaces may be small (Keele et al., 2008).

Early restriction of the virus may limit infection thereby providing time for adaptive immunity to expand, react, and clear the virus. However, many questions related to restricting mucosal replication remain. First, the half-life of virus in the mucosa is unknown. Second, more information is needed on target cell density and turnover, including efficiency of broadcasting infection to neighboring cells and distal tissue sites. Third, target cell restriction is likely very important; while it is known that activation status and cellular restriction factors can regulate susceptibility, additional information is needed. Lastly, triggering mucosal innate immunity may be a means of helping to prevent transmission.

The EUROPRISE Consortium is bringing together scientists from the microbicide and vaccine fields to develop a coordinated approach to HIV-1 prevention. The Initiative for Multipurpose Prevention Technology is an umbrella for government agencies, product developer, providers, researchers and advocates to work in an integrated development framework for products (devices, microbicides, and vaccines) that are designed to address multiple sexual and reproductive health needs, including prevention of unwanted pregnancy; prevention of STIs, including HIV-1; and/or prevention of other RTIs such as bacterial vaginosis, or urinary tract infections(PATH/CAMI, 2010). Future vaccine trials are likely to take place in the context of ARV-based PrEP and/or microbicide use. Microbicide-vaccine combinations may: 1) provide protection during the immunization period, 2) reduce infectious challenge, 3) lengthen the eclipse phase,providing time for the immune response to expand and respond, 4) boost localized immunity (virus/antigen), 5) broaden localized immunity through protected exposure, 6) bridge intermittent microbicide use, 7) cover resistant virus, or 8) provide better protection than either modality alone.

8. Can (protected) mucosal exposure influence immune response?

A study that exposed macaques to the vaginal formulation of TFV rectally either 15 min or 2 h prior to virus challenge yielded impor-

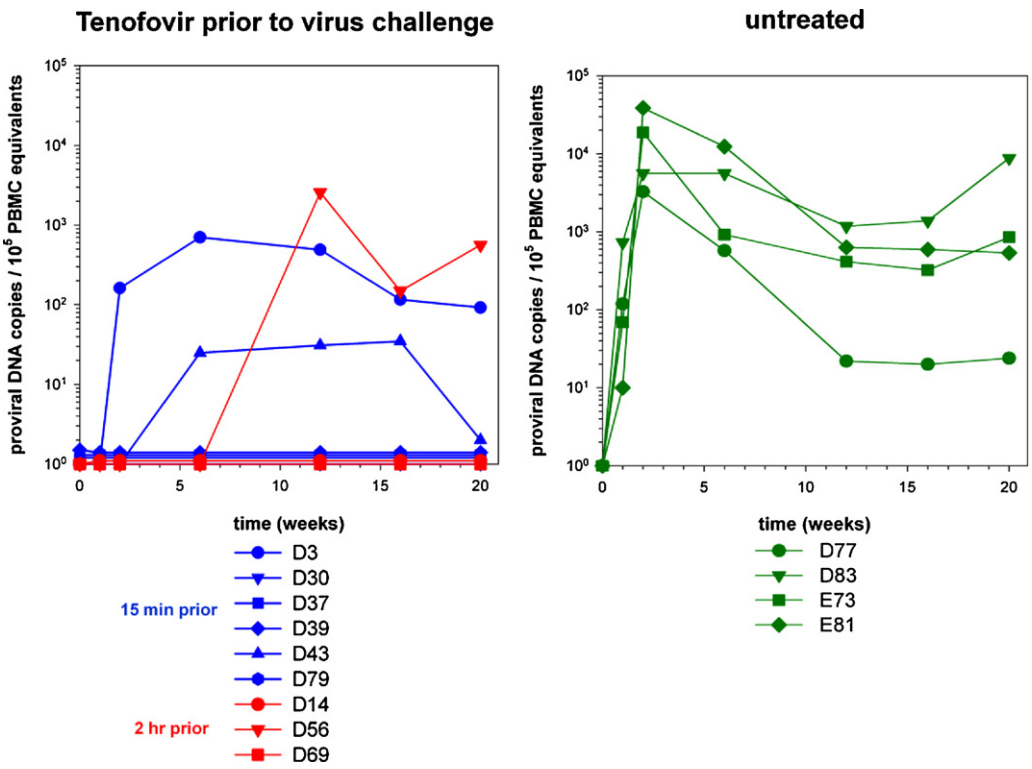

Fig. 2. Rectal exposure of TFV protected a high proportion of macaques against subsequent acquisition of SIV by rectal transmission compared with untreated control animals (Cranage et al., 2008).

tant information on cellular immunity as a marker of microbicide efficacy. Six out of the nine animals receiving TFV were protected from SIV. Four of the protected animals demonstrated T cell immunity responses against the virus despite the fact that they tested negative for virus, demonstrating that a microbicide can provide T cell immunitythrough non-infectious exposure to viral particles (Cranage et al., 2008) (Fig. 2). This approach has yet to be tested in humans and should be built into future microbicide trials.

9. Can vaccine candidates be co-formulated with microbicides?

The HIV-1 envelope offers major targets for virus neutralizing antibodies, and the breadth of neutralization is increased by the trimeric structure of envelop proteins (Burton et al., 2005; Cherpelis et al., 2001; Huang et al., 2005; Lian et al., 2005; Srivastava et al., 2002). Because it would be most relevant to the virus type that predominates in the developing world, CN54gp140, a Clade C construct, was chosen for testing as a candidate vaccine antigen. This construct showed good stability up to 36 months, with the trimeric nature of the construct remaining unchanged even when stored at room temperature. The compatibility of the antigen with different delivery technologies, including gels, IVRs, and tablets was investigated.

9.1. Gels

The stability of gp140 in vaginal lavage alone (in control gel or buffer) or combined with one of two gel formulations (HEC or PRO2000) was analyzed. Experiments showed that, if formulated correctly, antigenicity of the protein was maintained for at least 24 h. Based on these results, a Carbopol-based gp140 formulation was tested intravaginally in rabbits. Nine doses were administered during a 3-month period; the rabbits showed good antibody responses to gp140, indicating that vaginal immunization may be possible (Cranage et al., 2009).

9.2. IVRs

gp140 formulated into rods that can be inserted into IVRs showed sustained release for 30 days. Sustained release was also achieved with two different microbicides released from the body of the IVRs (Fig. 3). These gel and IVR experiments show that the same technology can be configured to deliver vaccine antigens and microbicides.

9.3. Self-administered dosage forms

Tablets that could be vaginally administered showed sustained release of gp140 during an 8-hour period, with about 75% of the gp140 released. Stability testing showed good stability, with more than 80% of the gp140 remaining after 150 days at 37 °C.

10. Can mucosal vaccination boost localized immunity?

Macaque studies showed that a priming intramuscular dose of 100 μg CN54gp140 in AS01b resulted in a good systemic response; intravaginal boosts of 100 μg CN54gp140 in Carbopol gel maintained that response (Fig. 4). Mucosal antibody titres showed the intramuscular dose was unable tomaintain mucosal responses, but the intravaginal boosts did.

11. Can protected mucosal exposure boost localized immunity?

A recently initiated study is testing whether protected mucosal exposure can boost localized immunity. NHPs are being vaccinated with gp140, first with a nasal prime and then with an intramuscular

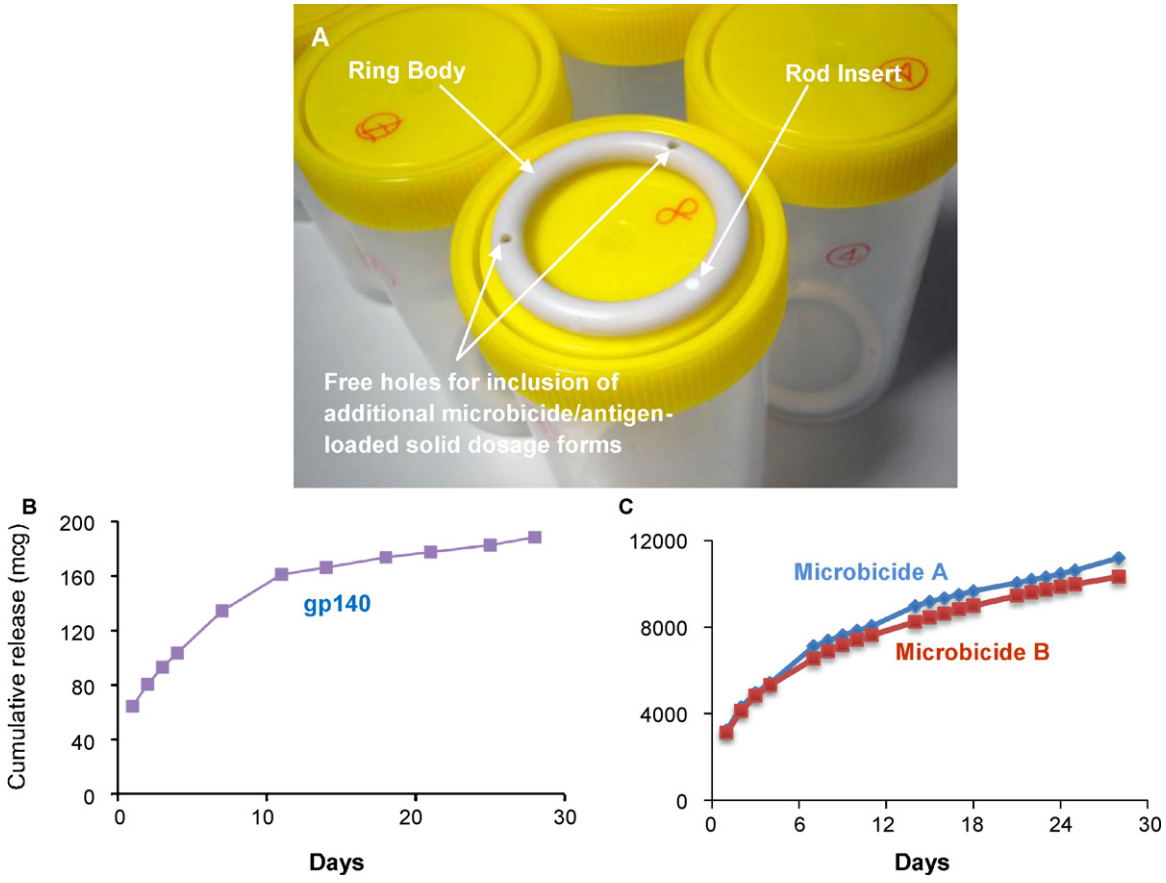

Fig. 3. IVR comprising single candidate antigen and two lead microbicide candidates. The antigen gp120 is formulated into the rod insert while the microbicides are formulated into the silicone elastomers.

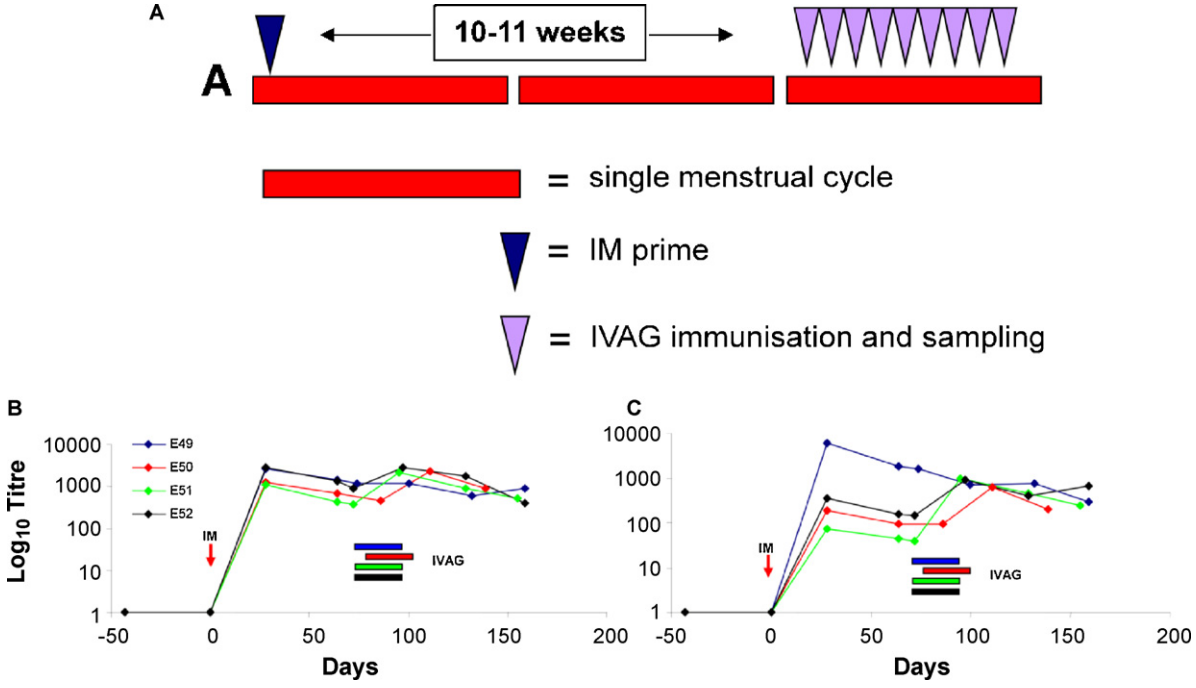

Fig. 4. Vaginal boosting of gp140 following intramuscular priming in macaques. Rhesus macaques were immunized with 100 μg CN54gp140 in ASO1b given by intramuscular injection followed by nine intravaginal doses (100 μg CN54gp140 in Carbopol gel) over one menstrual cycle (Panel A), serum gp140 specific IgG titers (Panel B) and IgA titres (Panel C).

boost. The NHPs will then be challenged with virus either in the presence or absence of a microbicide. Systemic and local immunity will be assessed at different time points (after immunization and after virus challenge both with and without microbicide). The study results will show which regimen provides the best protection and whether priming with a vaccine followed by protected exposure changes the profile of neutralizing antibodies.

12. Pathway to reversing the epidemic

Combined approaches will likely provide the best opportunity for preventionof HIV-1/STI transmission. PrEP or microbicides will likely be proven efficacious in the near future and could be combined with a partially effective vaccine to provide protection in the interim while a more effective vaccines and microbicides are developed.

13. Formulating microbicides to be nontoxic to vaginal and rectal mucosa

Several first generation candidate microbicides proceeded to pivotal clinical efficacy trials before being found to produce harm (i.e., greater incidence of HIV-1 acquisition(N9)), or possible trends toward harm (cellulose sulfate, C31G). These results revealed the pressing need to improve methods for detecting unacceptable toxic effects of candidate microbicides *before* they enter Phase III clinical trials.

Not only were early methods for assessing toxic effects of candidate microbicides insufficient to detect these toxicities, there was a paucity of data regarding excipients, components often assumed to be non-toxic on the basis of past experience with commercial vaginal products. But these excipients and products were never subjected to tests that might reveal toxic effects that are highly significant for microbicide products, namely increased susceptibility to HIV-1 infection. Moreover, most toxicity tests in animals are designed to monitor surrogates for susceptibility, rather than test directly for increased susceptibility to infection.

The first report of microbicide toxicity was by Phillips and Zacharopoulos (1998) who found that rectal application of N9 not only failed to protect mice against rectal transmission of HSV-2, but caused rapid exfoliation of sheets of epithelial cells. Within 5 min of rectal application, this detergent greatly increased susceptibility to infection. This report was an alarm bell for microbicide developers, and greatly increased efforts to detect and characterize toxic effects of candidate microbicides in animal models and clinical studies to screen candidate microbicides before they advanced to major clinical efficacy trials.

Vaginal susceptibility models (Cone et al., 2006; Galen et al., 2007) have helped provide a clear basis for understanding how N9 and C31G failed to protect against HIV-1 even though these detergents potently and rapidly blocked infection in vitro by enveloped viruses like HIV-1 and HSV-2. For example, in the mouse vaginal HSV-2 model, detergents protect (i.e., reduce susceptibility) only briefly (~5 min) but then cause a prolonged increase in susceptibility,which increases ~5 fold within 15 min and 12 h later every detergent tested to date increases susceptibility ~20–30 fold (Cone et al., 2006). Moreover, in the mouse/chlamydia model, a single vaginal application of CHX-2, a surface-active preservative/antiseptic, causes a 100-fold increase in susceptibility to Chlamydia 3 *days* after application (Achilles et al., 2002). Mouse models for HSV-2 and Chlamydia thus provide a way to detect toxicity *in vivo* that increases vaginal susceptibility to viral, as well as bacterial, STI pathogens. Arguably, toxic effects that increase susceptibility are more directly significant for vetting candidate microbicides than surrogate toxicities such as colposcopic abnormalities or other biomarkers hoped to correlate with susceptibility.

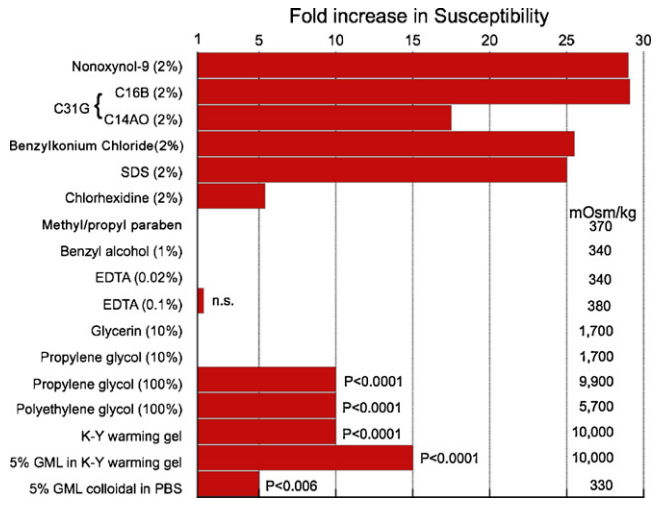

Fig. 5. Increase in susceptibility following single exposure in the mouse/HSV-2 model following single dose of test agent delivered to mouse vagina and a low-dose HSV inoculums delivered 12 h later (Cone et al., 2006).

Most protection tests in mouse models use high-dose inocula, typically ≥10 ID_{50} that infect most or all the control animals (Tuyama et al., 2006; Bourne et al., 2003; Bernstein et al., 2003; Maguire et al., 2001; Zeitlin et al., 1997; Whaley et al., 1993). If protective, the candidate reduces the fraction of animals infected. However, by infecting all or nearly all control animals, a high-dose inoculum eliminates the possibility of detecting toxic effects that increase ('enhance') susceptibility to infection. Thus, to detect toxic effects that increase susceptibility requires use of a low-dose inoculum that delivers 1 ID_{50} or less. In this case, if the candidate compound increases susceptibility, the fraction of animals infected by the low-dose, inoculum increases. The increase in susceptibility caused by the candidate can then be inferred from the fraction of control animals infected as a function of the dose of virus in the inoculum (the dose-response curve).

In developing the first generation of microbicides, considerable attention was paid to identifying candidate APIs demonstrating protective efficacy in animal models. However, less was done to accurately assess their toxic effects. In addition, essentially no toxicity tests of the excipients were performed in formulating first generation of microbicides, i.e., the "inactive" ingredients in the formulation such as gelling and thickening agents, preservatives, ionic composition and osmolality. Moreover, a commonly used study design has been to compare the active product to a different vehicle control gel (in most cases the HEC placebo gel (Tien et al., 2005)), a design that inevitably precludes detecting toxicity caused by excipients in the active formulation. Although often called "inactive ingredients", and widely considered to be benign, many excipients can cause toxicities. For example, highly hyperosmotic sexual lubricants cause rapid and marked shedding of rectal and colonic epithelial surfaces (Fuchs et al., 2007) and marked secretion of mucus in the slug mucosal irritation assay (Adriaens and Remon, 2008).

Fig. 5 summarizes results to date using the 'single exposure' mouse/HSV-2 model, in which a single dose of the test agent is delivered to the mouse vagina and a low-dose HSV-2 inoculum is delivered 12 h later, the time at which susceptibility rises to a maximum following a single dose of N9. The mice are pretreated with progestin (Depo Provera®) to thin the vaginal epithelium such that the entire surface of the epithelium is covered with living cells (comparable to the columnar epithelium of ectopy, endocervix, and upper tract in humans). The methods used to obtain results shown in Fig. 5 were described previously (Cone et al., 2006); Fig. 5

combines results reported in that publication with the recent excipient test results reported at Microbicides 2010, Pittsburgh, Poster 103 and Poster Discussion PD#3 "How Safe is Safe?").

Detergents (N9, C31G (Savvy), benzylkonium chloride, and SDS) all increased HSV-2 susceptibility about 20-30-fold. Viral inoculum could be reduced 20-30 fold and still infect the same fraction of animals as infected by the full inoculum in the control animals12 h after a single dose of these agents. For infections, such as HIV-1 and HSV-2 that are transmitted with low probability per coital event, this increase in susceptibility implies transmission probability per coital event increases 20-30 fold. CHX caused a 5-fold increase in HSV-2 susceptibility, less than the 100-fold increase it causes in the mouse/Chlamydia model, but still highly significant.

Two of the most frequently used preservatives, methyl/propyl paraben and benzyl alcohol caused no detected toxicity with this model. However, disodium EDTA, at the upper level of concentrations at which it is used as a preservative (0.1%), showed a non-significant trend toward toxicity. It is expected that higher concentrations of this metal ion (Ca^{++} and others) chelating agent causes peeling or shedding of epithelial surfaces.

Glycerol and propylene glycol, common excipients in mucosal products, showed no toxicity when applied at relatively low concentrations (10%), but caused significant toxicity (~10-fold increase in susceptibility) when applied at 100% (neat), at which both of these 'humectants' are highly hypertonic (5,700–9,900 mOsm/Kg). A recently marketed non-aqueous sexual lubricant, K-Y®Warming Jelly, is also hypertonic and causes similar toxicity.

Finally, GML in K-Y Warming Jelly has recently been shown to protect against SHIV in the monkey (Li et al., 2009), and 6 months of daily vaginal applications failed to elicit histologically detected toxicity in the female reproductive tract (Schlievert et al., 2008). However, GML has surfactant, emulsifier, membrane active, and penetration-enhancing actions, and when formulated in K-Y Warming Jelly (because GML is poorly solubilized in water) the combination caused significant toxicity (a ~15-fold increase in susceptibility). To detect whether GML alone contributed to toxicity, 5% GML was vigorously mixed with PBS to produce a colloidal suspension. This isotonic suspension also caused significant toxicity, a ~5-fold increase in susceptibility.

In summary, results obtained using the mouse/HSV-2 susceptibility models caution against using surface-active agents (CHX, GML), detergents, and highly hypertonic formulations. Methyl/propyl paraben, and benzyl alcohol, and low concentrations of EDTA appear acceptable as preservatives.

14. Conclusions

Prevention of HIV-1/STI transmission has been approached using non-specific broad-spectrum agents and more recently HIV-1 specificRTIs delivered in vaginal gels. Looking beyond traditional formulations and drugs, there are a number of new drugs, including biopharmaceuticals, nanoparticles, and combinations of mucosal vaccines and microbicides under active investigation. Vaginal delivery of these agents can be accomplished through gels, IVRs, films, and tablets. Experiments in animals suggest these approaches deserve further investigation.

The safety of microbicide formulations, including various excipients commonly used in vaginal dosage forms has been investigated mostly using methods unable to assess potential for the API or excipients to influence penetration of virus. Since early microbicides in some cases suggested increased infectivity of HIV-1 in Phase III clinical trials, new methods are required to determine how safe any API and its excipients are. The mouse/HSV-2 susceptibility model is one approach to assess potential APIs and excipients impacting potential HIV-1 transmission.

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Appendix A. Panel Discussion – Gaps Identified and Future Prospects for Microbicide Delivery

Jim Turpin: I would like to ask each panelist to highlight any novel or out-of-the-box approaches that he/she feels have emerged from this session. I'm challenging the panelists to look ahead to future opportunities while also identifying potential traffic jams and roadblocks.

Kate Morrow: The first roadblock that I see is that I am the only one conducting behavioral microbicide formulation research. There need to be other researchers working on this as well. My work is unique in that it is preclinical work that involves human subjects. Many of the challenges that I encounter are logistics-related, including receiving the active formulations and developing similar placebos. Another issue is that behavioral studies take time and patience. Of course, no obstacle would be insurmountable if we had unlimited funds.

David Friend: The biggest challenge that we face in product development is that there are a limited number of drugs available to work with and when you work intensely with some of these drugs, you realize that they are not ideal. So, a lot of times we are doing the best we can with what we have. It would be advantageous if there were some other compounds available that were more amenable to pharmaceutical development. In our case, we are working mostly with TFV and UC781; it would be nice if we could also work with some of the other drugs currently available for the treatment of HIV.

Richard Cone: It is known that women with bacterial vaginosis (BV) are at greater risk of acquiring HIV. Let's look at this another way, which is that women who have *Lactobacilli* as their dominant vaginal flora are at lower risk for HIV. Studies of BV have shown that it is a condition that comes and goes frequently. Women with BV typically have two episodes per menstrual cycle, so the trials that have studied BV risk factors greatly underestimate the risk of not having *Lactobacilli*. We need a variety of approaches to attack this epidemic and one of those could be preventing BV.

Craig Hendrix: I think what is missing is the proof-of-concept study between the Phase I and Phase III studies. There needs to be a bridge that explores the exposure-response relationship. To do this, biomarkers for both toxicity and efficacy are needed. The bold idea is to develop a human HIV challenge, perhaps using inactivated virus, as this would allow for the testing of different doses and different frequencies of exposure and would provide the type of rich data that is currently lacking.

Kevin Whaley: If we are going to develop multi-purpose prevention technologies that address a variety of reproductive and sexual health indications, the formulation job will be significant as it may involve different combinations of different types of molecules. I have been encouraged by the presentations on rings. Rings can be modular which will allow administration of different drugs without requiring extensive formulation work. A recent editorial in *Contraception* by David Grimes emphasized the need for long-term "forgettable" contraception. With rings, there is less reliance on user compliance. The further we can move away from relying on end users, perhaps the greater efficacy these products will have.

Justin Hanes: One method, even if highly effective, is not optimal because women want choices. So I think we should be working on a number of different approaches to HIV prevention. Secondly, establishment of HIV infection actually occurs fairly infrequently during intercourse with an infected person. What this says to me is that,

normally people are fairly well protected against HIV. The question is then, what occurs in the exceptional case in which a person becomes infected? Better understanding of the circumstances that make infection possible would help us in designing drug therapies or other ways to prevent HIV infection. Some people assume that the virus will enter the body andthey focus on stopping it at the cellular level, but, to me, that seems akin to letting the Trojan horse into the castle and hoping you can protect the people in the castle. It seems to me that we should be focusing on barrier methods to keep that Trojan horse out in the first place.

Robin Shattock: I would like to see additional work on PK/PD in animals and humans to better understand drug penetration and the amount of drug needed. Currently, there are some microbicide candidates that absorb well and some that do not and it is not known which is better; it would be helpful if studies were undertaken to address this question. That said, the answer to whether the poorly absorbed or the better absorbed NNRTIs work better will likely come from Phase III clinical trials. My concern is if these trials fail to show protection against HIV there may not be a lot of funder momentum to continue testing microbicides. This field is in a transition phase, with many people still grieving over the lack of effectiveness of non-anti-retroviral microbicides that could have been sold over-the-counter. The field needs to focus on the future and work to demonstrate proof-of-concept. Showing that a microbicide candidate is effective will provide the momentum for investment in early stage concepts and in the exploration of combination microbicides, which may address the resistance issue.

Gustavo Doncel: The panel has stated well the needs of the field and I agree with all of the comments made thus far. As I stated yesterday, there are still unanswered questions regarding transmission that hinder our ability to identify the most appropriate and efficacious microbicide. Until we can clearly state the amount of drug needed, where it is needed and how long it must remain, it will be difficult to develop a microbicide that we can have confidence in to prevent HIV transmission. That said, candidate microbicide development work cannot be halted to concentrate on understanding the biology of HIV transmission. Similarly, proof-of-concept is needed, but development work cannot stop because there is this need. Studying failed microbicide candidates is an important element of development as it will help us determine what led to those failures. I agree with Craig's comments on using a surrogate model to build the preclinical assessment and early clinical assessment to enable better selection of candidate microbicides. This should occur in parallel with the continued development and testing of the most advanced microbicide candidates. This field is still relatively new, but the evidence base is growing and we are in a much better position now to develop a safe and efficacious microbicide than we were just 10 years ago. This symposium has contributed to the understanding of the state of microbicide formulation.

Jim Turpin: One overarching theme that I have heard in these comments is the need for proof-of-concept, either in developing improved animal models to allow for better assessment of candidate microbicides or at the human level to show a true level of protection. Another common theme is the need for additional data, both basic data and data that will support a better approach to microbicide development. From this discussion, I've realized that this is a unique field as it is not basic science and it is not drug discovery, but is somewhere in between the two. There are data gaps, but these are being filled as the field moves forward. Another common theme is the multi-approach. This is more than simply combining two microbicides, and includes concepts such as combining a microbicide and a vaccine, combining an anti-STI drug and a microbicide, or combining a contraceptive with a microbicide. This theme also encompasses formulating a single microbicide in different ways, as a gel, tablet, film or ring. In considering all of these themes, one overarching issue is funding for all of these activities. I would like to ask the panel and the audience to respond to these themes.

David Friend: One challenge is that there are a limited number of groups working in microbicide formulation. It has been a bit of a struggle to identify universities and organizations that can develop the products that we want to develop. The few groups that have the capacity to do this formulation work end up being loaded up with many projects, so progress on any one individual project can be overwhelmed. The bottom line is that additional technical capability is needed.

Kate Morrow: One lesson that I have learned working in this field is the need for a multi-disciplinary approach. I am a psychologist and I am working with a bioengineer, a chemist, and a medical anthropologist in my behavioral microbicide work. It is also important that all of the people working on the different types of formulations collaborate.

Alan Stone: When the British Medical Research Council began working on microbicides, a decision was made to study mechanism of infection. The aim was to work logically and scientifically, but there was also this urgent target of protecting women from HIV infection, so another component of the work was empirical testing. During the past two decades, the difficulties have multiplied because this is an extremely challenging problem to solve. We should not lose sight of the fact that our aim is to develop a microbicide that is simple, inexpensive, safe and user friendly. A luminally active microbicide, if one can be developed, circumvents the need to worry a lot about the different pathways of infection. My concern is that things have become more and more complicated. We should aim to achieve protection in the simplest way possible.

Florian Hladik: There was a comment about the need for more basic data. This type of data tends to be generated by small laboratories that are newly recruited to the microbicide field. In my experience, it took years to obtain cellulose sulfate to test in our mucosal model. The HIV/AIDS field has been driven by the NIH repository which provides investigators with reagents free-of-charge. This type of free exchange of microbicides and other related substances is invaluable; it might be a good idea to advertise the availability of these products to entice basic laboratories not currently working with microbicides to do so.

Jim Turpin: The NIH makes efforts to add microbicide candidates to the repository. One concern, however, is that an investigator using a drug from the repository may do something to muddy the regulatory path. At the same time, efforts are being made to add new drugs to the repository to make them available to investigators. Recent additions include CCR5 inhibitors, tenofovir, and PMPA. Also, HEC placebo, which can be used in animal models or in mano testing, was recently added to the repository. The NIH wants to encourage research, but there are concerns about the regulatory pathway for these drugs. NIH also funds microbicide research through the Microbicide Innovation Program.

Badri Saxena: Dave, is there any reason silicone cannot be used in a ring? Second, a comment: women also need contraception and protection from other STIs, so combination microbicides are very important.

David Friend: There is no materials reason why silicones cannot be used in IVRs other than the fact that there are a limited number of suppliers. Silicone did not prove to be the right matrix material for UC781, but silicone can be used with other microbicides. The dapivirine ring is silicone-based.

Jim Turpin: Thank you to Gustavo Doncel, David Friend, and Sanjay Garg and to CONRAD for supporting this workshop. I judge the success of a meeting or workshop by the number of new ideas generated, and this workshop has generated a number of new ideas.

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