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A quantitative multiplex nuclease protection assay reveals immunotoxicity gene expression profiles in the rabbit model for vaginal drug safety evaluation

Raina N. Fichorova [a,*], Kevin Mendonca [a], Hidemi S. Yamamoto [a], Ryan Murray [a], Neelima Chandra [b], Gustavo F. Doncel [b]

[a] Laboratory of Genital Tract Biology, Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA
[b] CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA, USA

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ABSTRACT

Any vaginal product that alters the mucosal environment and impairs the immune barrier increases the risk of sexually transmitted infections, especially HIV infection, which thrives on mucosal damage and inflammation. The FDA-recommended rabbit vaginal irritation (RVI) model serves as a first line selection tool for vaginal products; however, for decades it has been limited to histopathology scoring, insufficient to select safe anti-HIV microbicides. In this study we incorporate to the RVI model a novel quantitative nuclease protection assay (qNPA) to quantify mRNA levels of 25 genes representing leukocyte differentiation markers, toll-like receptors (TLR), cytokines, chemokines, epithelial repair, microbicidal and vascular markers, by designing two multiplex arrays. Tissue sections were obtained from 36 rabbits (6 per treatment arm) after 14 daily applications of a placebo gel, saline, 4% nonoxynol-9 (N-9), and three combinations of the anti-HIV microbicides tenofovir (TFV) and UC781 in escalating concentrations (highest: 10% TFV + 2.5%UC781). Results showed that increased expression levels of toll-like receptor (TLR)-4, interleukin (IL)-1β, CXCL8, epithelial membrane protein (EMP)-1 ($P < 0.05$), and decreased levels of TLR2 ($P < 0.05$), TLR3 and bactericidal permeability increasing protein (BPI) ($P < 0.001$) were associated with cervicovaginal mucosal alteration (histopathology). Seven markers showed a significant linear trend predicting epithelial damage (up with CD4, IL-1β, CXCL8, CCL2, CCL21, EMP1 and down with BPI). Despite the low tissue damage RVI scores, the high-dose microbicide combination gel caused activation of HIV host cells (SLC and CD4) while N-9 caused proinflammatory gene upregulation (IL-8 and TLR4) suggesting a potential for increasing risk of HIV via different mechanisms depending on the chemical nature of the test product.

Abbreviations: ANOVA, analysis of variance; ARV, anti-retroviral; BPI, bactericidal permeability increasing protein; BWH, Brigham and Women's Hospital; CCL, chemokine C-C motif ligand; CCL, chemokine C-C motif ligand; CD, cluster of differentiation; CONRAD, Contraceptive Research and Development Program; COX, cyclooxygenase; CXCL, chemokine C-X-C motif ligand; EMP, Epithelial membrane protein; FFPE, formalin-fixed paraffin-embedded; HEC, hydroxypropyl cellulose; HIV, human immunodeficiency virus; HPA, high power areas; IACUC, Institutional Animal Care and Use Committee; IL, interleukin; LPS, lipopolysaccharides; MCP, macrophage chemotactic protein; mRNA, messenger ribonucleic acid; N-9, Nonoxynol-9; NAP, neutrophil attractant protein; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; NP-3a Def, Neutrophil defensin 3a; NRC, National Research Council; PAMP, pathogen associated molecular patterns; PBS, phosphate buffered saline; PHS, policy on humane care and use of laboratory animals; PTGR, prostaglandin reductase; PTGS, prostaglandin-endoperoxide synthase; qNPA, quantitative nuclease protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; RVI, rabbit vaginal irritation; SD, standard deviation; SELL, L-Selectin; SELP, P-Selectin; SLC, secondary lymphoid-tissue chemokine; TFV, tenofovir; TLR, toll-like receptor; TNF, tumor necrosis factor; USDA, United States Department of Agriculture; USFDA, United States Federal Drug Administration; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

* Corresponding author at: Laboratory of Genital Tract Biology, Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women's Hospital and Harvard Medical School, 221 Longwood Av, RF468, Boston, MA 02115, USA. Fax: +1 617 713 3018.
E-mail address: rfichorova@rics.bwh.harvard.edu (R.N. Fichorova).

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Introduction

Topical microbicides are developed for reducing the sexual transmission of HIV-1 and other sexually transmitted infections. Recent reviews of topical microbicides to prevent the transmission of HIV (Fichorova, 2015; Pulaganti et al., 2014) define various stages in the effort to develop safe and effective microbicides. Initially, broad-spectrum detergents and polyanions were tried, but these products where either ineffective or enhanced HIV transmission. During this period there was also a rapid progress in antiretroviral therapy, which led to the current stage of development with products containing antiretroviral (ARV) compounds such as tenofovir (TFV), dapivirine and UC781. A systematic Cochrane review (Obiero et al., 2012) examined the outcomes of randomized clinical trials of all anti-HIV microbicides conducted between 2004 and 2011. Eight broad-spectrum microbicides were tested during this time and none of them were successful. Conversely, one product containing tenofovir was tested and shown to be partially protective (Abdool Karim et al., 2010).

The lack of effectiveness of vaginal anti-HIV microbicides has much been blamed on unwanted local inflammatory reaction and tissue damage (Fichorova, 2015; Pulaganti et al., 2014). These safety concerns are especially raised for anti-HIV topicals because while inflammation is part of the normal response to clear bacterial and other viral infections, it is not efficient in clearing HIV infection, and in fact, it increases the risk of HIV infection. Inflammatory cytokines and chemokines drive and activate CD4+ cells making them more susceptible to HIV infection, and once the cells are infected they also stimulate HIV replication via nuclear factor kappa B. At the same time interferon-stimulated innate immune responses contribute to aborting HIV infection even though HIV has developed mechanisms to evade these protective responses making them less efficient in clearing infection (Goff, 2013; Rustagi and Gale, 2014). Furthermore, inflammation causes tissue damage and opens inter-cellular tight junctions thus allowing more viruses to penetrate the epithelium and reach HIV host cells in the submucosa (Mesquita et al., 2009). Therefore, guidelines for product development have evolved to include rigorous preclinical evaluation of candidate formulations using biomarkers of mucosal inflammation (Fichorova, 2004; Fichorova et al., 2001) driven by the premise that to be effective the anti-HIV microbicides should not alter the integrity of the mucosal barrier and must lack immunotoxicity defined as any significant upregulation of pro-inflammatory genes or suppression or anti-inflammatory genes and immunoregulatory genes in comparison to a placebo that may: 1) facilitate HIV transepithelial penetration and replication in infected cells, and/or 2) impair protective responses against concurrent bacterial and viral infections.

Traditionally animal models have been used for the preclinical safety screening of microbicides *in vivo*. The animal models that have been used include mouse (Lozenski et al., 2011, 2012; Xu et al., 2013), pig (Banerjee et al., 2011b), monkey (Banerjee et al., 2011a) and rabbit (Banerjee et al., 2011a; Strazza et al., 2011). Among the animal models used to screen the compatibility of candidate anti-HIV topical microbicides with the vaginal epithelial barrier, the rabbit vaginal irritation (RVI) model is the one recommended by the USFDA for safety evaluation of vaginal products prior to phase I clinical trials (Center for Drug Evaluation and Research, 1995). For more than 40 years this model has served to determine toxicity using traditional pathology endpoints like epithelial erosion, vascular congestion, edema and leukocyte infiltration (Eckstein et al., 1969). Unlike other small rodent models it allows testing of full strength formulations and full-dose volumes of 1.0–1.5 ml without leaking. Furthermore, the simple columnar epithelium of the rabbit's abdominal vagina offers higher testing sensitivity for a reasonable margin of safety selection prior to human use. At the same time, the rabbit model is much less expensive than primate models for evaluating vaginal microbicide compounds and formulations. In addition to systemic toxicity, the classic RVI version includes a gross pathology and histology evaluation of the vaginal tissue. However, in this classic version the RVI model has failed to predict the unwanted side effects of anti-HIV microbicides prior to entering clinical trials. A well-studied example is nonoxynal-9 (N-9), which was considered safe based on classic RVI endpoints and preclinical evaluation but failed to protect and even caused harm and increased risk of HIV infection in efficacy clinical trials (Van Damme et al., 1998, 2000, 2002). Vaginal formulations with up to 4% N-9 consistently received RVI scores that are 'acceptable' for human use and this experience led to the need to improve the RVI testing, including cytokines and other markers of mucosal inflammation and immunotoxicity (Hillier et al., 2005).

To improve the rabbit model and make it more relevant to mucosal safety we have established a refined RVI version, including markers of leukocyte infiltration and proinflammatory activation such as enumeration of CD45-positive cells, and levels of cytokines in vaginal washings including IL-1α and β, IL-6 and IL-8, which correlated with findings in human studies (Fichorova, 2004; Fichorova et al., 2011; Mauck et al., 2013; Trifonova et al., 2006, 2007). Using immunocytochemistry we identified additional markers of immunoinflammatory activation such as NF-κB p60, VCAM-1, e-selectin and ICAM-1 (Trifonova et al., 2007). This work was duplicated and successfully confirmed by a number of other studies conducted in the rabbit model and also in mice, rats, guinea pigs and primates (Alt et al., 2009; Costin et al., 2011; Zhong et al., 2012). However, the wide and efficient use of the refined RVI model has been hampered by limited availability of immunoassays and specific antibodies against rabbit proteins, as well as by the semi-quantitative aspects of the immunohistochemical type of analyses.

To overcome these limitations we applied a multiplex mRNA platform based on quantitative nuclease protection assay (qNPA), previously applied for assessment of human inflammatory genes (Leviton et al., 2012; Trifonova et al., 2009). We assessed expression of multiple rabbit inflammation-associated genes with known sequences with the intent to distinguish more subtle pathological gradients of epithelial tissue damage and inflammation in comparison to the crude histopathological scoring system and to discriminate between microbicide gel formulations of different strength and mechanism of action.

Materials and methods

Rabbit treatment. Young adult reproductive age nulliparous New Zealand White Hra:(NZW)SPF rabbits (Covance Research Products, Chantilly, VA) (5–8 months old, body weight 2.5 kg ±20%) were used for this study. Test compounds included a normal saline solution (Center Medical Supply, Portage, Michigan), the placebo gel HEC (Hydroxyethylcellulose, 250 HX PHARM, Hercules, Wilmington, Delaware), a 4% N-9 gel (Ortho Options Conceptrol Vaginal Contraceptive Gel, available over-the-counter) and prototype combination gels containing UC781, and TFV (all prepared and provided by University of Utah, Salt Lake City, Utah (Cost et al., 2012)). The combination gels were prepared in the following three proportions: 1) low dose combo gel (0.25% UC781 and 1% TFV), 2) middle dose combo gel (0.75% UC781 and 3% TFV); and 3) high dose combo gel (2.5% UC781 and 10% TFV). TFV was obtained from Gilead Sciences (Foster City, California). UC781 was prepared by custom synthesis by Regis Technologies (Morton Grove, Illinois). UC781 used in the gels was micronized (D50 of 2.2:m). We evaluated N-9 as a classic example of a microbicide that passed the traditional RVI test but failed due to inflammatory damage. It is a widely accepted control for vaginal inflammatory responses that led to clinical failure. It had previously shown mild irritation in the RVI model and thus was well suited for the goal of our investigation which was to discover biomarkers of more subtle perturbations that would be missed in Phase I trials by routine clinical and colposcopic examinations. In addition we evaluated combination prototype gels with TFV and UC781 in 3 different proportions. The combination of more than one ARV in the vaginal delivery system offers additional mechanisms of efficacy by targeting more than one event in the viral replication cycle (Pirrone et al., 2011). Combination gels with TFV and UC781