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# Biomarkers of leukocyte traffic and activation in the vaginal mucosa

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**Abstract**

Development of novel vaginal spermicides and anti-human immunodeficiency virus (HIV) microbicides requires careful assessment of their potential to recruit and activate CD4+ HIV-1 host cells in the female genital tract mucosa, two events that facilitate HIV-1 infection. Leukocyte traffic and activation are mediated by proinflammatory cytokines and chemokines, e.g. interleukin (IL)-1, IL-6 and IL-8, which have been detected in vaginal secretions in association with epithelial damage and infections. These proinflammatory mediators, however, have bidirectional, destructive as well as beneficial, effects on the mucosal barrier, and may be counterbalanced by endogenous inhibitors. Here we propose additional biomarkers for the evaluation of compound-induced cervicovaginal mucosal inflammation. Displaying different temporal patterns of detection, the levels of soluble E-selectin, vascular adhesion molecule-1, CD14 and myeloperoxidase in vaginal secretions reflected the mucosal leukocyte reaction to proinflammatory compounds being evaluated for safety in an improved rabbit vaginal irritation model. These biomarkers, which were also detected in human vaginal secretions, may be used to enhance the characterization of mucosal safety of vaginally applied compounds, both in animal as well as clinical studies.

**Keywords:** *Anti-HIV-1 microbicides, CD14, myeloperoxidase, vaginitis, VCAM-1, E-selectin*

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## Introduction

Global human immunodeficiency virus (HIV)-1 infection rates among women continue to rise dramatically each year (Simon et al. 2006). An emerging strategy for limiting the spread of sexually transmitted HIV-1 infection is the development of topical anti-HIV-1 microbicides (Phillips & Maguire 2002, Stone 2002, Shattock & Moore 2003, McGowan 2006). For such topical agents to be effective they must preserve or enhance the natural mucosal barrier of the female genital tract. Numerous

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studies link inflammatory conditions in the female genital tract to increased risk of sexually transmitted HIV-1 infection and transmission (reviewed in Quinn 1996, Fichorova 2004, Dhawan & Mayer 2006), suggesting the need for monitoring the proinflammatory potential of vaginal products under development.

The inflammatory reaction initiated by irritating compounds may promote HIV-1 transmission by eroding the protective mucosal epithelial layers and release of proinflammatory cytokines (e.g. interleukin (IL)-1α and IL-1β) and chemokines (e.g. IL-8, interferon-γ-inducible protein (IP)-10 and macrophage inflammatory protein (MIP)-3α) capable of recruiting neutrophils and CD4+ HIV-1 host cells such as monocytes, T cells and dendritic cells (Fichorova et al. 2001b, Fichorova et al. 2004, Fichorova et al. 2005, Trifonova et al. 2006). This mucosal inflammatory reaction would facilitate penetration of cell-free and cell-associated HIV-1 virus and infection of mucosal immune cells with subsequent cytokine-mediated HIV-1 replication, viral shedding and increased risk of HIV-1 transmission (Alfano & Poli 2005, Hillier et al. 2005, Dhawan & Mayer 2006).

Findings from recent experimental and clinical studies suggest that the development of successful microbicides and safe vaginal products requires a careful evaluation of their impact on the vaginal mucosal barrier (Fichorova et al. 2001b, Catalone et al. 2005, Fichorova et al. 2005, Hillier et al. 2005, Cone et al. 2006, Fletcher et al. 2006, Trifonova et al. 2006, Cummins et al. 2007, Galen et al. 2007). It has been postulated that product selection should be based on preclinical safety assessment in a relevant animal model and non-invasive biomarkers of tissue injury and inflammation in clinical trials (Lard-Whiteford et al. 2004). The rabbit vaginal irritation test (RVI) has been widely used for more than 30 years as the US Food and Drug Administration (FDA)-required model for safety evaluation of vaginal products. However, in its original version, the RVI test is based on standard histopathological evaluation, which is not adequate for detection of inflammatory conditions favouring HIV-1 infection of mucosal tissues (Eckstein et al. 1969, Doncel et al. 2004, Fichorova et al. 2004). A compelling example of this model's deficiencies is the failure of the first compound to be assessed as a topical anti-HIV-1 agent, nonoxynol-9 (N-9), which passed the standard RVI test and phase I/II safety trials but, with frequent use, increased HIV-1 transmission in phase II/III clinical trials (reviewed in Hillier et al. 2005).

In a refined version of the RVI model we have demonstrated that IL-1, IL-6 and IL-8 may be used as biomarkers of epithelial disruption induced by proinflammatory compounds (Fichorova et al. 2004). The same cytokines in human cervicovaginal lavage (CVL) have also been linked to irritating vaginal exposures (Fichorova et al. 2001b). However, these mediators reflect early events in the inflammatory cascade that may be blocked by natural inhibitors such as soluble receptors and receptor antagonists (O'Neil & Greene 1998, Trifonova et al. 2006). Moreover, depending on the cellular and environmental context, they may have pleiotropic or opposing functions ranging from propagation of the inflammatory reaction and tissue destruction to cell proliferation and tissue repair (Foster 2001, Sugawara et al. 2001). These properties complicate the interpretation of cytokine profiles and their use as biomarkers of vaginal product safety. Therefore, markers specific for tissue inflammatory events downstream from the cytokine/chemokine cascade would be a useful addition to current tools for monitoring the vaginal mucosa immunoinflammatory status.

Responding to this need, the present study identifies non-invasive soluble markers of vascular and leukocyte activation that correlate with levels of tissue damage and influx of potential HIV-1 host cells following vaginal product use in the refined RVI model. To validate these markers against standard histopathological criteria, the study utilized N-9 and other well-characterized spermicidal and microbicidal surface active agents that have shown a broad range of toxic and proinflammatory responses *in vitro* and *in vivo* (Krebs et al. 2002, Fichorova et al. 2004, Howett & Kuhl 2005, Cone et al. 2006).

## Materials and methods

### *Reagents*

Benzalkonium chloride (BZK), sodium dodecyl sulfate (SDS) and carboxymethylcelluose (CMC) were purchased from Sigma Co. (St Louis, MO, USA). Nonoxynol-9 (N-9) was a kind gift from Personal Products Company (Skillman, NJ, USA). The following mouse anti-human rabbit cross-reactive monoclonal antibodies were used: anti-E-selectin from Sigma-Aldrich (St Louis, MO, USA), anti-CD14 and anti-vascular adhesion molecule (VCAM)-1 from Biosource (Camarillo, CA, USA), anti-nuclear factor (NF)-κB/p65 from Chemicon (Temecula, CA, USA) and anti-phospho-Fos (pS374) from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Conjugated mouse monoclonal antibodies specific for rabbit CD45 (FITC) and CD4 (PE) and fluorochrome isotype controls were purchased from Research Diagnostics, Inc. (Flanders, NJ, USA).



### *Animal protocol*

All experimentation with live animals was conducted within the provisions of the Animal Welfare Act (Public Law 99-198), the National Research Council (NRC), Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS), the 'Guide for the Care and Use of Laboratory Animals' (1996), the Health Research Extension Act of 1985 Public Law 99-158 (11/20/86), and United States Department of Agriculture (USDA) regulations. The work was performed under an Eastern Virginia Medical School Institutional Animal Care and Use Committee (IACUC)-approved protocol with additional approval from the Standing Committee on Animals of the Harvard Medical School Office for Research Subject Protection.

Adult (3–4 kg), nulliparous, non-pregnant, New Zealand white female rabbits were dosed intravaginally with 1 ml of active formulation (2% N-9, SDS, or BZK in 4.5% CMC) or vehicle control (4.5% CMC) as previously described (Fichorova et al. 2004). Three rabbits per treatment group were dosed on three consecutive days. CVLs were collected prior to dose application at baseline (BL), at 24 and 48 h from BL, and on two consecutive days after the last dose application (72 and 96 h from BL). The lavage was performed by intravaginal flushing with 5 ml of 37°C saline using a 12-cm flexible catheter (polyethylene tubing; Fisher Scientific, Hampton, NH, USA). Cellular debris was removed by centrifugation and the CVL supernatants were frozen at −70°C until used for immunoenzyme detection of soluble markers. After the last CVL was collected at 96 h from BL, the rabbits were euthanized and vaginal tissues processed for immunohistochemical evaluation. Paraffin-embedded

tissue sections from the upper, mid, and lower portions of the abdominal vagina were processed for immunohistochemistry.

### *Human vaginal lavage samples*

To determine whether molecular markers detected in rabbit lavage are also detectable in human samples, CVL samples obtained from healthy volunteers participating in a CONRAD-funded microbicide study (Fichorova et al. 2004) were tested after IRB approval was obtained from the clinical site (WIRB Johns Hopkins University, Baltimore, MD, USA) and approved by the Brigham and Women's Hospital IRB for Human Subject Research. For this study, CVLs from 24 sexually active subjects with no genital infection and a normal Nugent score ($<7$) obtained in 10 ml saline prior to product use were used for detection of soluble products of cell activation and vascular markers. The cellular portion was removed by centrifugation at 500*g* and the supernatant frozen at $-70^{\circ}$C until used in the immunoassays described below.

### *Immunohistochemistry*

Immunohistochemical analysis of rabbit tissue sections was performed with an alkaline phosphatase biotin–streptavidin amplification system (BioGenex, San Ramon, CA, USA) and Fast Red Substrate System (Dako, Carpinteria, CA, USA), or immunofluorescence as previously described (Fichorova et al. 2001a). Deparaffinization was performed in xylene and ethyl alcohol following standard procedure while antigen retrieval was performed in an EDTA-based EZ-AR2 solution and EZ-Retriever™ oven (BioGenex) following the manufacturer's instructions. Slides were assessed and microphotographs taken using an Olympus BX60 microscope and Q-color 3™ Digital camera (Olympus America, Inc., Melville, NY, USA) with Image-Pro Plus 5.1.0.20 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

### *Immunoenzyme assays*

Levels of soluble (s) VCAM-1, sE-selectin, myeloperoxidase (MPO) and sCD14 in rabbit CVLs were determined by Searchlight multiplex technology (Pierce Biotechnology Group, Boston, MA, USA) and enzyme-linked immunosorbent assay (ELISA) as previously described (Fichorova et al. 2004). Soluble mediators were measured in human CVLs using the following immunoassays: a multiplex E-selectin and VCAM-1 assay read by Electrochemiluminescence Imager 2400 (both from Meso Scale Discovery, Gaithersburg, MD, USA); sCD14 Quantikine® (R&D Systems, Minneapolis, MN, USA) and MPO (Assay Designs, Ann Arbor, MI, USA) ELISAs read by Victor$^{2}$™ 1420 Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA). The lower limit of detection of each assay was determined as mean signal $+2$ SD of the zero standard read from the standard curve. Statistical analysis was performed using GraphPad Prism 4.0. Paired Student's *t*-tests were used to compare baseline versus treated samples and a $p$ value of $<0.05$ value was considered significant.

## Results

### *Tissue findings*

The immunohistochemical and morphological observations of the stained vaginal tissue sections at the end of the study period revealed that the vaginal mucosa was intact in the CMC-treated rabbits with single leukocytes randomly spread throughout the tissue. The tissues from BZK-, N-9- and SDS-treated rabbits, however, showed various degrees of epithelial damage, vascular dilatation, leukocyte infiltration and immunocytochemical signs of cell activation (Figure 1). BZK ranked as the most irritating agent followed by N-9 and SDS by both morphological and immunohistochemical criteria. The vaginal epithelial lining of all BZK-treated rabbits was

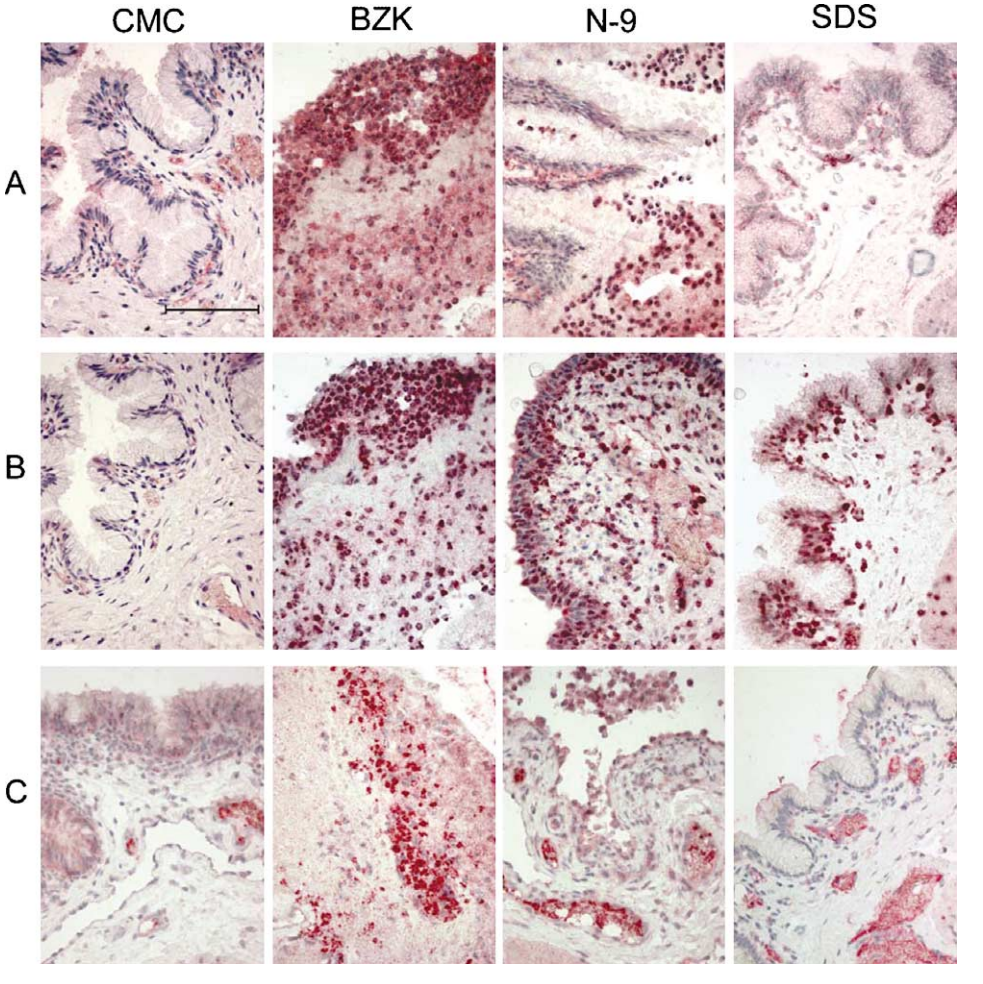


Figure 1. Representative photomicrographs comparing immunohistochemical findings for E-selectin (A), vascular adhesion molecule (VCAM)-1 (B) and CD14 (C) in full-thickness paraffin-embedded vaginal tissue sections from carboxymethylcelluose (CMC), benzalkonium chloride (BZK), nonoxynol-9 (N-9) and sodium dodecyl sulfate (SDS) (left to right) treated animals. Positive reaction is visualized by red substrate; haematoxylin counterstain; scale bar = 25 μm with the exception of BZK (A and C) where scale bar = 12.5 μm.

completely disrupted and massive leukocyte infiltrations were observed in the eroded mucosa. In the N-9-treated rabbits the vaginal epithelium was eroded and multiple intra- and submucosal leukocytes infiltrates and dilated blood vessels were found. Although the vaginal epithelium was intact in the SDS-treated rabbits, numerous leukocytes were present within the epithelial and subepithelial layers, accompanied by distended blood vessels.

E-selectin-positive staining was present in the lumen of blood vessels in all vaginal tissue samples, regardless of inflammatory condition or vascular distension (Figure 1A). While no positive leukocyte infiltrates were found in the CMC- and SDS-treated rabbits, except for some single positive cells in the lamina propria, E-selectin was expressed by infiltrating submucosal leukocytes in N-9- and BZK-treated rabbits.

The immunostaining for VCAM-1 in the same tissues also showed distinct patterns among the different treatment groups (Figure 1B). A faint positive endothelial cell staining and no positive leukocytes were observed in the tissues from animals in the CMC-treated control group. In contrast, all vaginal tissues from the BZK-treated rabbits showed massive VCAM-1-positive leukocyte infiltrates. Strong VCAM-1-positive leukocytes were abundant throughout the submucosa of all N-9-treated animals showing also positive staining in the endothelium of dilated blood vessels. SDS-treated rabbits displayed VCAM-1-positive vascular endothelium in all distended blood vessels and a number of VCAM-1-positive leukocytes in the lamina propria. Staining for CD14 showed positive leukocytes, which were predominantly localized within blood vessels in CMC- and SDS-treated tissues, but were abundant within extravascular granulomatous formations in the BZK- and N-9-treated tissues (Figure 1C).

Activation of NF-κB was examined by immunohistochemical detection of the nuclear localization sequence of the NF-κB p65 subunit, which is exposed only after dissociation of NF-κB from its cytoplasmic inhibitor IκB (Van Den Brink et al. 2000). The same antibody was used to demonstrate proinflammatory activation in human and rabbit vaginal epithelial cells *in vitro* and *in vivo* (Fichorova et al. 2001b, Doncel et al. 2004, Fichorova et al. 2005). The most representative patterns in each treatment group are presented in Figure 2A. A weak positive reaction was observed in the rare subepithelial leukocytes and epithelial cells in the CMC-treated vaginal tissues. Positive staining for activated p65 NF-κB was abundant in leukocytes residing in large inflammatory infiltrates of the submucosal tissue in BZK-treated rabbits. All N-9-treated vaginal tissue samples displayed abundant NF-κB-positive intraepithelial and subepithelial leukocytes whereas fewer positive leukocytes were observed in the SDS-treated vaginal tissue sections.

In addition to activated NF-κB, the activated (phosphorylated) form of the c-Fos subunit of the transcription factor AP-1, was also detected in leukocyte infiltrates of BZK- and N-9-treated tissue and in single intraepithelial or submucosal leukocytes present in SDS-treated vaginal tissue (Figure 2B). No activated c-Fos was detectable in the CMC-treated group.

The patterns of vascular adhesion molecules expression and NF-κB and c-Fos activation correlated with the abundance of CD45- and CD4-positive leukocytes detectable by immunofluorescence in tissue sections from BZK-, N-9- and SDS-treated rabbits (Figure 3). While the CMC-treated tissue was CD45- and CD4-negative, numerous CD45- and CD4-positive cells were present in the BZK- and

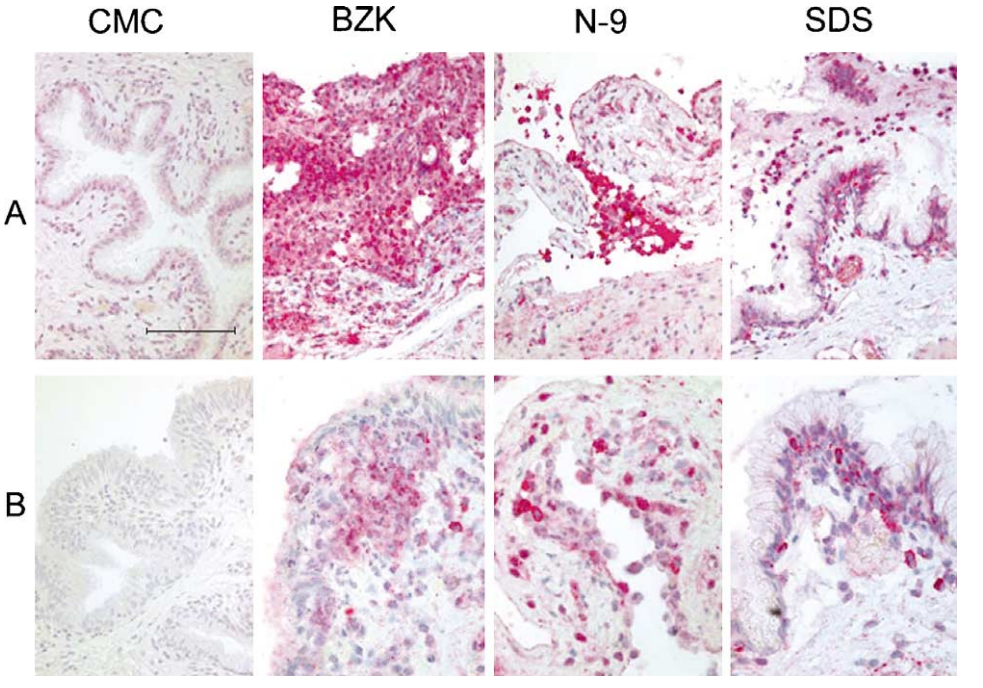


Figure 2. Immunohistochemical staining of nuclear factor (NF)-κB (A) and c-Fos (B) in full-thickness paraffin-embedded vaginal tissue sections from carboxymethylcelluose (CMC), benzalkonium chloride (BZK), nonoxynol-9 (N-9) and sodium dodecyl sulfate (SDS) (left to right) treated animals. Positive reaction is visualized by red substrate; haematoxylin counterstain; scale bar = 25 μm for CMC (A and B) and scale bar = 12.5 μm for BZK, N-9 and SDS (B).

N-9-treated vaginal tissue. Fewer positive CD45 and CD4 cells were found scattered within the epithelium and lamina propria in the SDS-treated vaginal tissue.

*Soluble markers*

The kinetic of soluble vascular and leukocyte activation markers measured in the rabbit CVLs is presented in Figure 4. In most rabbits, the baseline CVL levels of sE-selectin, VCAM-1, CD14 and MPO were close to the assay lower limit of detection. In the BZK-treated group, significant increases ($p < 0.001$) of all four markers were detected 96 h after the first dose (2 days after the treatment was discontinued). In contrast to sVCAM-1, MPO and CD14, which reached their highest levels at that time, sE-selectin showed a transient early peak, 24 h after the first BZK dose. VCAM-1, in turn, was the marker with the highest increase (almost two logs).

N-9 induced similar marker dynamics, with sVCAM-1, MPO and CD14 increases at 72 h and peaks at 96 h after the first dose; however, the differences did not reach statistical significance due to a higher variability among the rabbits of this group. Unlike the BZK treatment group, the N-9-treated group did not show significantly increased E-selectin levels at the 24 h time points after the first dose.

A lower magnitude of sE-selectin increase and earlier transient peaks of sVCAM-1, MPO and CD14 levels were observed in the SDS-treated rabbits. Changes seemed

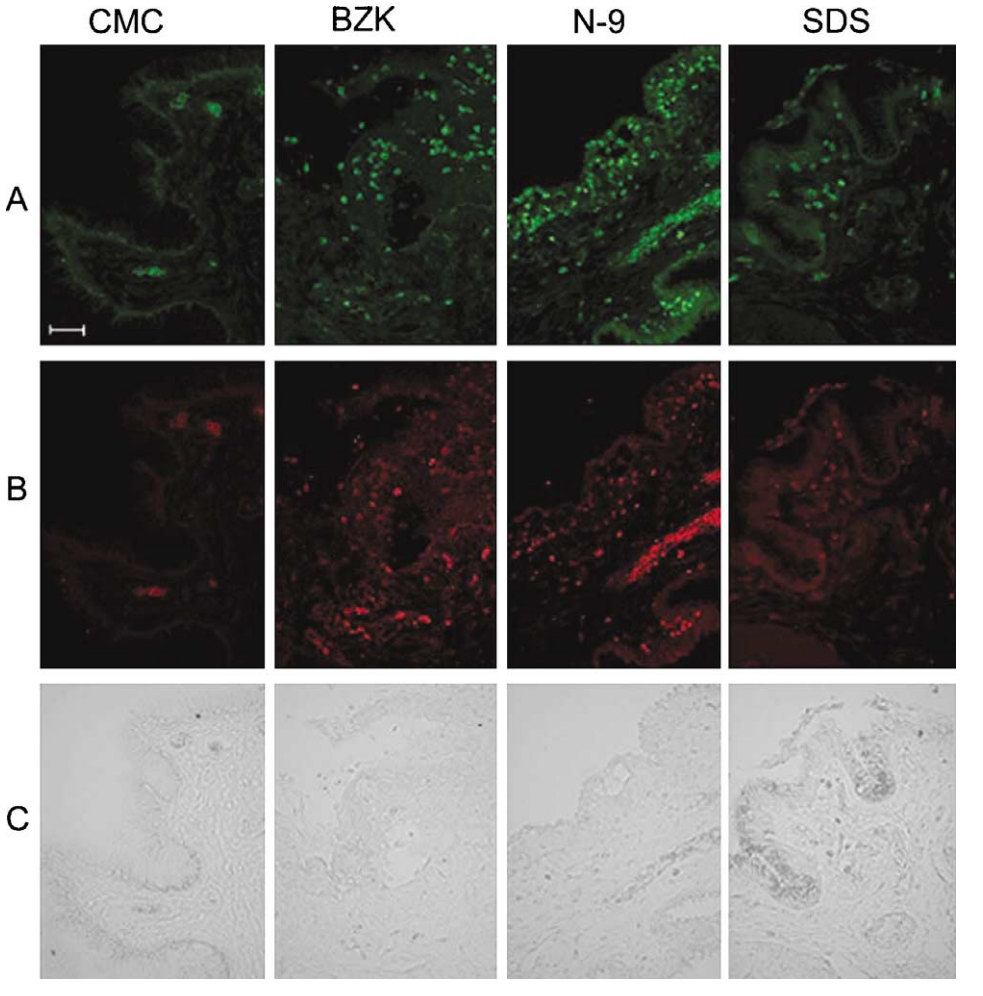


Figure 3. Representative photomicrograph of dual immunofluorescent staining demonstrating the presence of CD45 (green, A) and CD4 (red, B) positive cells in lamina propria of tissue sections from carboxymethylcelluose (CMC), benzalkonium chloride (BZK), nonoxynol-9 (N-9) and sodium dodecyl sulfate (SDS) (left to right) treated rabbits; scale bar = 25 μm. (C) Bright field.

more prominent at the 72 h time point but did not reach statistical significance. No significant increase over baseline was found in the CMC-treated rabbits.

Similarly to rabbit baseline CVLs, human CVLs from healthy women contained low but well-detectable levels of sE-selectin and sVCAM-1 that were close to the assay lower limit of detection. They also contained high levels of sCD14 and MPO that were reproducibly detectable in all samples (Figure 5).

## Discussion

This study demonstrates, for the first time, the biologically significant presence of soluble E-selectin and VCAM-1 in the female genital tract and confirms the presence of soluble CD14, previously detected in human vaginal swab samples (Fichorova et al.

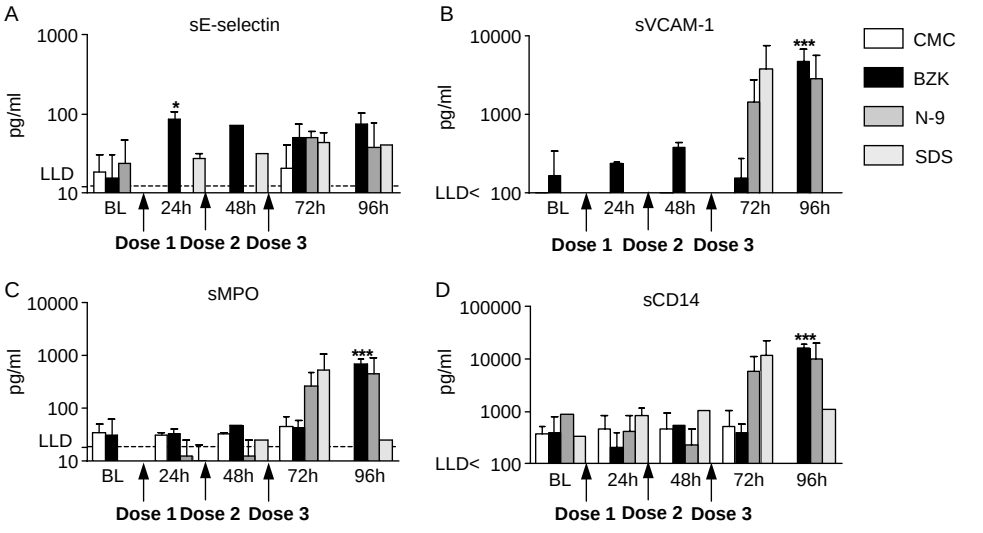


Figure 4. Detection of soluble (s)E-selectin (A), soluble vascular adhesion molecule (sVCAM)-1 (B), myeloperoxidase (sMPO) (C) and sCD14 (D) in rabbit vaginal lavage collected at five time points: before (at study baseline, BL), during (24 and 48 h from BL) and after (72 and 96 h from BL) daily vaginal administrations of carboxymethylcelluose (CMC), benzalkonium chloride (BZK), nonoxynol-9 (N-9) and sodium dodecyl sulfate (SDS). Each dose administration is marked by a black arrow. Results represent means and SEM from duplicate Searchlight measurements in cervicovaginal lavages from three rabbits per treatment group. Dotted line represents lower limit of detection (LLD) of assay.



2002). It also adds new evidence for the role of endogenous vaginal soluble MPO, which has been studied as an exogenous supplement intended to suppress bacterial vaginosis (Lichtenwalner et al. 2000). Our study is the first to compare levels of these soluble biomarkers with mucosal tissue signs of leukocyte activation.

It has been shown that E-selectin mediates early endothelial adhesion of polymorphonuclear leukocytes (PMNs) and monocytes, while VCAM-1 plays a predominant role in the late monocyte adhesion events (Carlos & Harlan 1994). The soluble forms of VCAM-1 and E-selectin can be generated by proteolytical cleavage of the transmembrane forms and have been identified in culture supernatants of endothelial cells as well as in human serum and cerebrospinal and synovial fluids of patients with multiple sclerosis and rheumatoid arthritis (Matsuda et al. 1995, Kitani et al. 1998, Xyni et al. 2000, Garton et al. 2003). Levels of sVCAM-1 and sE-selectin in plasma and urine have been proposed for monitoring disease activity and vascular change in patients with chronic inflammatory syndromes associated with endothelial dysfunction, e.g. systemic lupus erythematosus, rheumatoid arthritis and vasculitis (Blann et al. 1995, Littler et al. 1997, Molad et al. 2002, Mansoor et al. 2004). In the human female genital tract, VCAM-1 and E-selectin are abundant in cancer tissues (Coleman & Stanley 1994), but rarely and weakly expressed in the normal cervix and vagina (Johansson et al. 1999).

The increased levels of sE-selectin and sVCAM-1 in the rabbit CVLs were associated with the presence of dilated blood vessels and increased number of VCAM-1-positive leukocytes. The persistence of increased sE-selectin and/or sVCAM-1 levels agreed with the higher number of CD45- and CD4-positive lymphocytes in BZK- and N-9-treated tissue as compared with SDS-treated tissue.

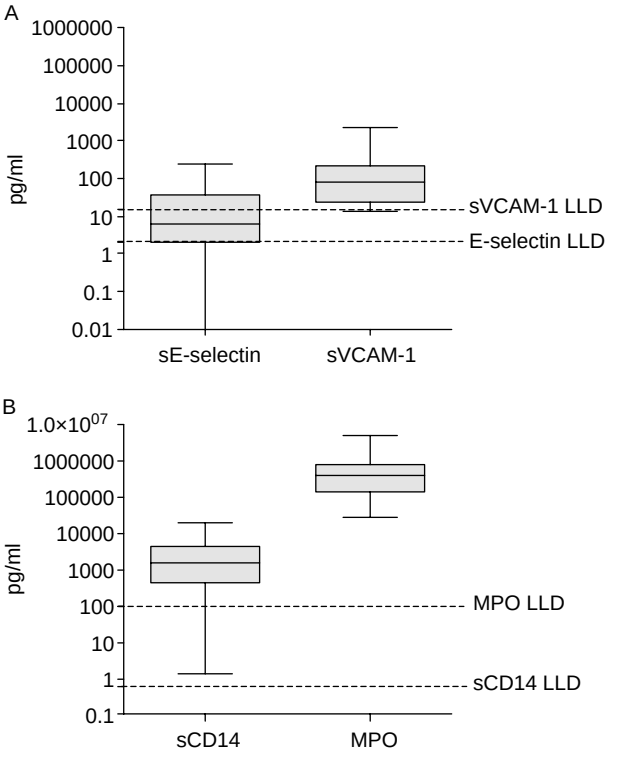


Figure 5. Box plots representing baseline levels of vascular adhesion molecules (A) and leukocyte activation markers (B) in human cervicovaginal lavage (CVLs) from healthy subjects ($n = 24$). The line in the middle is the median; the box extends from the 25th to the 75th percentile. The error bars (whiskers) extend down to the lowest and up to the highest value. Dotted line represents lower limit of detection (LLD) of assay. MPO, myeloperoxidase; VCAM, vascular adhesion molecule.



These findings are in agreement with previous ranking of BZK, N-9 and SDS based on cell toxicity and inflammatory cytokine responses *in vitro* and in the rabbit model (Krebs et al. 2002, Fichorova et al. 2004). Furthermore, NF-κB and c-Fos activation in leukocyte infiltrates, observed by immunohistochemical analysis of rabbit vaginal tissues, correlated with CVL levels of leukocyte-specific activation markers MPO and CD14 measured at the time of tissue collection.

CD14 is expressed by multiple leukocyte types and is essential for toll-like receptor (TLR)-mediated innate immune responses (Fujihara et al. 2003, Triantafilou & Triantafilou 2005). Soluble CD14 is present in CVL secretions and in semen and it enhances the response of vaginal epithelial cells to Gram-negative bacterial components (Fichorova et al. 2002). Levels of sCD14 in vaginal secretions were shown to correlate with HIV-1 shedding in the female genital tract (Lawn et al. 2000) suggesting its relevance as a biomarker of vaginal barrier function and increased risk of HIV-1 infection. MPO is released into the phagosome during the degranulation process, being responsible for the bactericidal properties of PMNs (Klebanoff 2005). It may also have HIV-1-suppressive effects (Klebanoff 2005). Thus, while marking the presence of an active inflammatory reaction with multiple HIV-1-promoting consequences, as an innate immune factor MPO may actually have protective antiviral effect on its own.

Based on the premise that biomarker detection in cervicovaginal secretions reflects a sequence of mucosal inflammatory events, we propose a kinetic model for compound-induced vaginal leukocyte traffic and activation (Figure 6). In this model, the cascade of immune activation starts with cytokines (e.g. IL-1) that have been previously identified in vaginal secretions of rabbits following exposure to N-9 and BZK (Fichorova et al. 2004). IL-1 released from damaged epithelial cells triggers NF-κB and c-Fos activation (Fichorova et al. 2005), which in turn lead to upregulation of

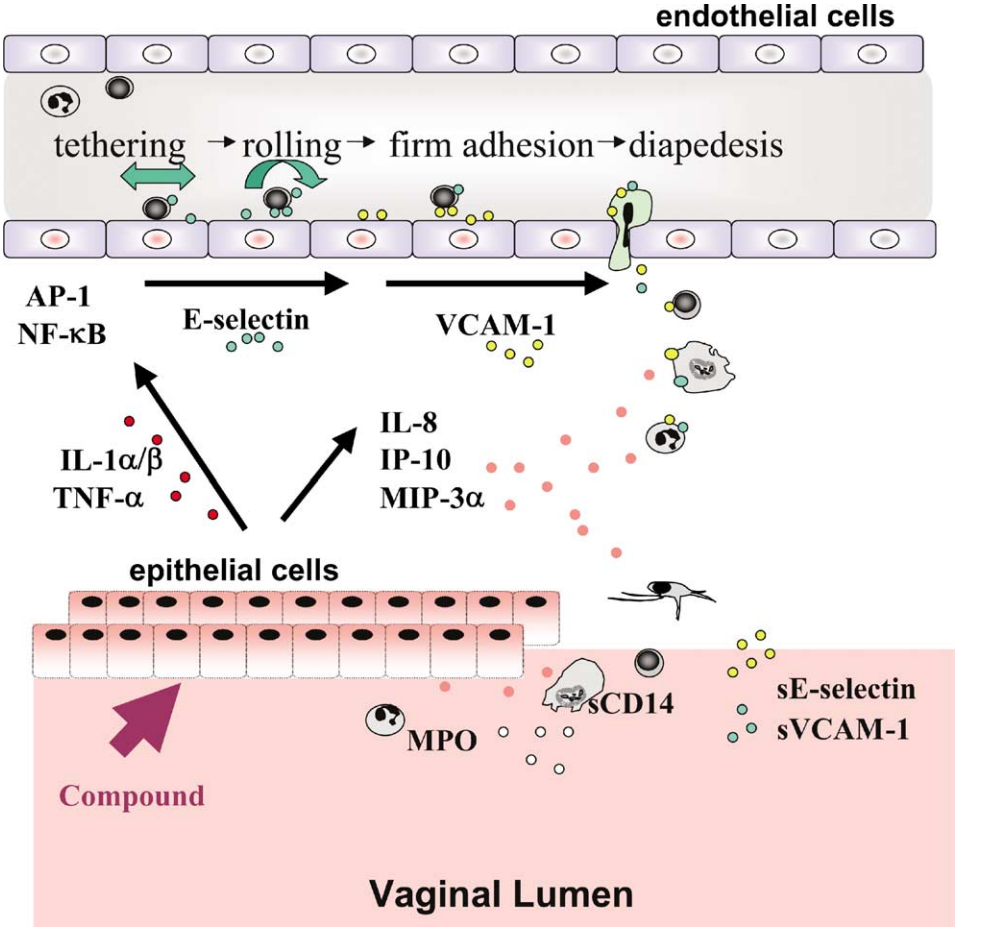


Figure 6. Schematic summary of the key events of endothelial cell activation and leukocyte mucosal infiltration in response to compound-mediated cervicovaginal epithelial irritation. The inflammatory cascade starts with release of interleukin (IL)-1 and other cytokines by damaged or stressed epithelial cells, followed by nuclear factor (NF)-κB and AP-1-mediated induction of endothelial vascular adhesion molecules and leukocyte trafficking and activation at the site of injury. The transendothelial migration involves a complex sequence of molecular and cellular events. The adhesion molecule E-selectin is involved in the early stages of leukocyte tethering and attachment to the endothelium, while VCAM-1 promotes late events such as firm adhesion and vascular diapedesis. The transmigrating leukocytes move up the chemokine gradient generated by the activated epithelium releasing more cytokines and shedding soluble (s) E-selectins, VCAM-1, CD 14 and/or myeloperoxidase (MPO) into the vaginal secretions. TNF, tumour necrosis factor; MIP, macrophage inflammatory protein; IP, interferon-γ-inducible protein; VCAM, vascular adhesion molecule.

E-selectin and VCAM-1 (Collins et al. 1995, Ahmad et al. 1998, Lee et al. 2005). E-selectin and VCAM-1 expressed by the mucosal endothelium and CD45-positive cells mediate the step-wise progression of leukocyte migration across the vascular wall (Carlos & Harlan 1994, Salmi & Jalkanen 2005). Migrating leukocytes and activated endothelium become the source of soluble adhesion molecules released into vaginal secretions. The leukocyte migration process is facilitated by chemokines (e.g. IL-8, MIP-3α, IP-10) expressed by vaginal epithelial cells when exposed to irritating compounds, as shown in *ex vivo* reconstructed vaginal epithelium studies (Trifonova et al. 2006). In addition, IL-8 leads to degranulation and release of MPO (Segura et al. 1998) and upregulation of CD14 on monocytes. This cascade of events is reflected by an increase of the soluble biomarkers E-selectin, VCAM-1, MPO and CD14 in vaginal washes.

We demonstrate in this study that metrics of vascular and leukocyte activation, non-invasively detectable in vaginal lavage samples, can predict the effects of surface active agents with a broad range of toxic and proinflammatory effects such as BZK, N-9 and SDS. The mildest of those detergents, SDS, is currently under development as a microbicidal spermicide (Howett & Kuhl 2005). Although abandoned as a vaginal microbicide candidate, N-9 continues to be present in genital hygiene and spermicide over-the-counter products (Trifonova et al. 2006). Thus, understanding the mechanisms of action of surface active agents for vaginal application continues to be of public health importance. Future studies should determine possible mechanisms, by which non-detergent classes of vaginal microbicides impact the vaginal mucosal barrier, and may add more detail in the sequel of immunoinflammatory events and biomarker release, schematically presented in Figure 6. Ideally, a vaginal microbicide product should be evaluated by a combination of biomarkers and functional assays reflecting various aspects of molecular, cellular and host–pathogen interactions (Doncel et al. 2004, Fichorova et al. 2005).

This study proposes novel soluble non-invasive markers of vaginal leukocyte influx and activation and validates their utility in the context of the FDA-recommended RVI model by comparison with results from standard pathology and immunochemistry. The results from this study support the use of soluble MPO, CD14, E-selectin and VCAM-1 in animal models designed to evaluate mucosal inflammation induced by vaginal products and pave the way for future clinical validation of these markers. Further studies should examine their physiological variation in the context of sexual and genital hygiene practices and their use for monitoring undesirable side-effects of various classes of vaginal products in women at risk for HIV-1 and other sexually transmitted infections.



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## References

Ahmad M, Theofanidis P, Medford RM. 1998. Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-alpha. Journal of Biological Chemistry 273:4616–4621.

Alfano M, Poli G. 2005. Role of cytokines and chemokines in the regulation of innate immunity and HIV infection. Molecular Immunology 42:161–182.

Blann AD, Herrick A, Jayson MI. 1995. Altered levels of soluble adhesion molecules in rheumatoid arthritis, vasculitis and systemic sclerosis. British Journal of Rheumatology 34:814–819.

Carlos TM, Harlan JM. 1994. Leukocyte–endothelial adhesion molecules. Blood 84:2068–2101.

Catalone BJ, Miller SR, Ferguson ML, Malamud D, Kish-Catalone T, Thakkar NJ, Krebs FC, Howett MK, Wigdahl B. 2005. Toxicity, inflammation, and anti-human immunodeficiency virus type 1 activity following exposure to chemical moieties of C31G. Biomedical Pharmacotherapy 59:430–437.

Coleman N, Stanley MA. 1994. Characterization and functional analysis of the expression of vascular adhesion molecules in human papillomavirus-related disease of the cervix. Cancer 74:884–892.

Collins T, Read M, Neish A, Whitley M, Thanos D, Maniatis T. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB Journal 9:899–909.

Cone RA, Hoen T, Wong X, Abusuwwa R, Anderson DJ, Moench TR. 2006. Vaginal microbicides: detecting toxicities in vivo that paradoxically increase pathogen transmission. BMC Infectious Diseases 6:90.

Cummins JE Jr, Guarner J, Flowers L, Guenthner PC, Bartlett J, Morken T, Grohskopf LA, Paxton L, Dezzutti CS. 2007. Preclinical testing of candidate topical microbicides for anti-HIV-1 activity and tissue toxicity in a human cervical explant culture. Antimicrobial Agents and Chemotherapy 51:1770–1779.

Dhawan D, Mayer KH. 2006. Microbicides to prevent HIV transmission: overcoming obstacles to chemical barrier protection. Journal of Infectious Diseases 193:36–44.

Doncel GF, Chandra N, Fichorova RN. 2004. Preclinical assessment of the proinflammatory potential of microbicide candidates. Journal of Acquired Immune Deficiency Syndrome 37(Suppl. 3):S174–S180.

Eckstein P, Jackson MCN, Millman N, Sobrero AJ. 1969. Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys. Journal of Reproductive Fertility 20:85–93.

Fichorova RN, Desai PJ, Gibson F, Genco CA. 2001a. Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells. Infection and Immunity 69:5840–5848.

Fichorova RN, Tucker L, Anderson DJ. 2001b. The molecular basis of nonoxynol-9 induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. Journal of Infectious Diseases 184:418–428.

Fichorova RN, Cronin A, Lien E, Anderson DJ, Ingalls RR. 2002. Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of TLR-4-mediated signaling. Journal of Immunology 168:2424–2432.

Fichorova RN. 2004. Guiding the vaginal microbicide trials with biomarkers of inflammation. Journal of Acquired Immune Deficiency Syndrome 35:S1–S8.

Fichorova RN, Bajpai M, Chandra N, Hsiu JG, Spangler M, Ratnam V, Doncel GF. 2004. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biological Reproduction 71:761–769.

Fichorova RN, Zhou F, Ratnam V, Atanassova V, Jiang S, Strick N, Neurath AR. 2005. Anti-human immunodeficiency virus type 1 microbicide cellulose acetate 1,2-benzenedicarboxylate in a human in vitro model of vaginal inflammation. Antimicrobial Agents and Chemotherapy 49:323–335.

Fletcher PS, Wallace GS, Mesquita PM, Shattock RJ. 2006. Candidate polyanion microbicides inhibit HIV-1 infection and dissemination pathways in human cervical explants. Retrovirology 3:46.

Foster JR. 2001. The functions of cytokines and their uses in toxicology. International Journal of Experimental Pathology 82:171–192.

Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H. 2003. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacology & Therapeutics 100:171–194.

Galen BT, Martin AP, Hazrati E, Garin A, Guzman E, Wilson SS, Porter DD, Lira SA, Keller MJ, Herold BC. 2007. A comprehensive murine model to evaluate topical vaginal microbicides: mucosal inflammation and susceptibility to genital herpes as surrogate markers of safety. Journal of Infectious Disease 195:1332–1339.

Garton KJ, Gough PJ, Philalay J, Wille PT, Blobel CP, Whitehead RH, Dempsey PJ, Raines EW. 2003. Stimulated shedding of vascular cell adhesion molecule 1 (VCAM-1) is mediated by tumor necrosis factor-alpha-converting enzyme (ADAM 17). Journal of Biological Chemistry 278:37459–37464.

Hillier SL, Moench T, Shattock R, Black R, Reichelderfer P, Veronese F. 2005. In vitro and in vivo: the story of nonoxynol 9. Journal of Acquired Immune Deficiency Syndrome 39:1–8.

Howett MK, Kuhl JP. 2005. Microbicides for prevention of transmission of sexually transmitted diseases. Current Pharmaceutical Design 11:3731–3746.

Johansson EL, Rudin A, Wassen L, Holmgren J. 1999. Distribution of lymphocytes and adhesion molecules in human cervix and vagina. Immunology 96:272–277.

Kitani A, Nakashima N, Izumihara T, Inagaki M, Baoui X, Yu S, Matsuda T, Matsuyama T. 1998. Soluble VCAM-1 induces chemotaxis of Jurkat and synovial fluid T cells bearing high affinity very late antigen-4. Journal of Immunology 161:4931–4938.

Klebanoff SJ. 2005. Myeloperoxidase: friend and foe. Journal of Leukocyte Biology 77:598–625.

Krebs FC, Miller SR, Catalone BJ, Fichorova R, Anderson D, Malamud D, Howett MK, Wigdahl B. 2002. Comparative in vitro sensitivities of human immune cell lines, vaginal and cervical epithelial cell lines, and primary cells to candidate microbicides nonoxynol 9, C31G, and sodium dodecyl sulfate. Antimicrobial Agents and Chemotherapy 46:2292–2298.

Lard-Whiteford SL, Matecka D, O'Rear JJ, Yuen IS, Litterst C, Reichelderfer P. 2004. Recommendations for the nonclinical development of topical microbicides for prevention of HIV transmission: an update. Journal of Acquired Immune Deficiency Syndrome 36:541–552.

Lawn SD, Subbarao S, Wright TC Jr, Evans-Strickfaden T, Ellerbrock TV, Lennox JL, Butera ST, Hart CE. 2000. Correlation between human immunodeficiency virus type 1 RNA levels in the female genital tract and immune activation associated with ulceration of the cervix. Journal of Infectious Disease 181:1950–1956.

Lee DK, Kang JE, Park HJ, Kim MH, Yim TH, Kim JM, Heo MK, Kim KY, Kwon HJ, Hur MW. 2005. FBI-1 enhances transcription of the nuclear factor-kappaB (NF-kappaB)-responsive E-selectin gene by nuclear localization of the p65 subunit of NF-kappaB. Journal of Biological Chemistry 280:27783–27791.

Lichtenwalner AB, Patton DL, Klebanoff SJ, Headley CM, Hillier SL. 2000. Vaginal myeloperoxidase and flora in the pig-tailed macaque. Journal of Medical Primatology 29:36–41.

Littler A, Buckley C, Wordsworth P, Collins I, Martinson J, Simmons D. 1997. A distinct profile of six soluble adhesion molecules (ICAM-1, ICAM-3, VCAM-1, E-selectin, L-selectin and P-selectin) in rheumatoid arthritis. Rheumatology 36:164–169.

Mansoor MA, Seljeflot I, Arnesen H, Knudsen A, Bates CJ, Mishra G, Larsen TW. 2004. Endothelial cell adhesion molecules in healthy adults during acute hyperhomocysteinemia and mild hypertriglyceridemia. Clinical Biochemistry 37:408–414.

Matsuda M, Tsukada N, Miyagi K, Yanagisawa N. 1995. Increased levels of soluble vascular cell adhesion molecule-1 (VCAM-1) in the cerebrospinal fluid and sera of patients with multiple sclerosis and human T lymphotropic virus type-1-associated myelopathy. Journal of Neuroimmunology 59:35–40.

McGowan I. 2006. Microbicides: a new frontier in HIV prevention. Biologicals 34:241–255.

Molad Y, Miroshnik E, Sulkes J, Pitlik S, Weinberger A, Monselise Y. 2002. Urinary soluble VCAM-1 in systemic lupus erythematosus: a clinical marker for monitoring disease activity and damage. Clinical and Experimental Rheumatology 20:403–406.

O'Neil LA, Greene C. 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects and plants. Journal of Leukocyte Biology 63:650–657.

Phillips DM, Maguire RA. 2002. The development of microbicides for clinical use to prevent sexually transmitted diseases. Current Infectious Disease Reports 4:135–140.

Quinn TC. 1996. Association of sexually transmitted diseases and infection with the human immunodeficiency virus: biological cofactors and markers of behavioral interventions. International Journal of STD & AIDS 7(Suppl. 2):17–24.

Salmi M, Jalkanen S. 2005. Cell-surface enzymes in control of leukocyte trafficking. Nature Reviews. Immunology 5:760–771.

Segura RM, Alegre J, Varela E, Marti R, Surinach JM, Jufresa J, Armadans L, Pascual C, Fernandez de Sevilla T. 1998. Interleukin-8 and markers of neutrophil degranulation in pleural effusions. American Journal of Respiratory & Critical Care Medicine 157:1565–1572.

Shattock RJ, Moore JP. 2003. Inhibiting sexual transmission of HIV-1 infection. Nature Reviews. Microbiology 1:25–34.

Simon V, Ho DD, Abdool Karim Q. 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet 368:489–504.

Stone A. 2002. Microbicides: a new approach to preventing HIV and other sexually transmitted infections. Nature Reviews. Drug Discovery 1:977–985.

Sugawara T, Gallucci RM, Simeonova PP, Luster MI. 2001. Regulation and role of interleukin 6 in wounded human epithelial keratinocytes. Cytokine 15:328–336.

Triantafilou M, Triantafilou K. 2005. The dynamics of LPS recognition: complex orchestration of multiple receptors. Journal of Endotoxin Research 11:5–11.

Trifonova RT, Pasicznyk JM, Fichorova RN. 2006. Biocompatibility of solid-dosage forms of anti-human immunodeficiency virus type 1 microbicides with the human cervicovaginal mucosa modeled ex vivo. Antimicrobial Agents and Chemotherapy 50:4005–4010.

Van den Brink GR, ten Kate FJ, Ponsioen CY, Rive MM, Tytgat GN, van Deventer SJH, Peppelenbosch MP. 2000. Expression and activation of NF-κB in the antrum of the human stomach. Journal of Immunology 164:3353–3359.

Xyni K, Rizos D, Giannaki G, Sarandakou A, Phocas I, Creatsas G. 2000. Soluble form of ICAM-1, VCAM-1, E- and L-selectin in human milk. Mediators of Inflammation 9:133–140.