L-Selectin and P-Selectin Are Novel Biomarkers of Cervicovaginal Inflammation for Preclinical Mucosal Safety Assessment of Anti-HIV-1 Microbicide

Running Title: L and P-selectin as biomarkers of mucosal inflammation

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A major obstacle thwarting preclinical development of microbicides is the lack of a validated biomarker of cervicovaginal inflammation. Therefore, the present study aims to identify novel noninvasive soluble markers in a murine model for assessment of microbicide mucosal safety. By performing cytokine antibody array analysis, we identified two adhesion molecules, L-selectin and P-selectin, which significantly increased when mucosal inflammation was triggered by Nonoxynol-9 (N9), the clinical trial-failed anti-HIV-1 microbicide candidate, in a refined murine model of agent-induced cervicovaginal inflammation. We found that L-selectin and P-selectin had obviously different patterns of detection than the two previously defined biomarkers of cervicovaginal inflammation, monocyte chemotactic protein 1 (MCP-1) and interleukine 6 (IL-6). These two soluble selectins correlated better than MCP-1 and IL-6 with the duration and severity of mucosal inflammation triggered by N9 and two approved proinflammatory compounds: benzalkonium chloride (BZK), sodium dodecyl sulfate (SDS), but not by two non-proinflammatory compounds: carboxymethylcellulose (CMC, microbicide excipients) and tenofivor (TFV, microbicide candidate). These data indicated that L-selectin and P-selectin can serve as additional novel cervicovaginal inflammation biomarkers for preclinical mucosal safety evaluation of candidate microbicides for the prevention of HIV and other sexually transmitted pathogens.
INTRODUCTION

The epidemic of human immunodeficiency virus type 1 (HIV-1), when combined with the lack of an effective vaccine, has created an urgent need for a female-controlled method to reduce the risk of HIV-1 transmission (35). Microbicides are defined as products that can be applied topically for the prevention of HIV-1 and other sexually transmitted infections by creating chemical, biological and/or physical barriers. As such, they may offer one of the most promising preventive interventions by their accessible cost, ready availability, wide acceptance and female-controlled use (35). To date, seven candidate microbicides have been tested in clinical trials. These included Nonoxynol-9 (N9), Savvy, cellulose sulphate, Carraguard, PRO 2000, BufferGel and tenofovir. While tenofovir showed some degree of preventive effects, the other products were shown to have no effect on HIV-1. Among them, cellulose sulphate and N9 were even shown to have a trend towards increased risk of HIV-1 infection (1, 35).

N9, a nonionic surfactant, was the first candidate microbicide to be tested in efficacy clinical trials. Early studies indicated that N9 was active against a range of bacteria and enveloped viruses in vitro, including HIV-1 and herpes simplex virus (HSV), and various N9 products, including COL-1492, were defined as generally safe by colposcopic examination in phase I and phase II clinical trials (30, 40, 41). However, clinical trials to evaluate the efficacy of COL-1492 (containing 52.5mg N9) in protecting against HIV-1 infection have generated disappointing outcomes. Specifically, N9 did not prevent HIV-1 infection when used at low frequency, and it even increased the risk of HIV-1 infection by frequent use (42). A series of following studies found that genital inflammation and the disruption of the cervicovaginal epithelium induced by microbicides are critical risk factors associated with the
incidence of HIV-1 infection (9, 13, 15, 16, 18).

In constructing a safety profile, much can be learned from the N9 story. First, microbicidal efficacy depends on striking a balance between specific activity and safety, and the absence of inflammatory events is a key issue for microbicidal safety. Second, colposcopic evaluation is an unreliable predictor of local inflammation. Finally, inflammation resulting from exposure to N9 has been detected by different methods, including histology, neutrophil count, and cytokine-chemokine release (18).

Although histology analysis could enable investigators to identify inflammation, it would also require biopsy specimens of vaginal or cervical tissue taken shortly before and after exposure to the test agent. In addition, this method is qualitative and not sensitive enough to determine mucosal inflammation without obvious tissue damage. Thus, a noninvasive, more sensitive and quantifiable method to describe the alterations caused by microbicidal agents with proinflammatory potential would be preferable (12). Many studies have used CVF as samples for the analysis of proteins, and correlations were found between different expression profiles and specific pathologies, resulting in the identification of biomarkers of these specific conditions and diseases (45). Many studies have used CVF as samples for the analysis of proteins, and correlations were found between different expression profiles and specific pathologies, resulting in the identification of biomarkers of these specific conditions and diseases (3, 28). Use of a biomarker in drug development can reduce the costs and time required to get a drug from discovery to market. Relevant research in the area of microbicide safety is yielding new assays and biomarkers which, if validated, will be essential to the rational selection of microbicide candidates for efficacy trials (29). Use of a biomarker in drug development can reduce the costs and time required to get a drug from discovery to market. Relevant research in the area of
microbicide safety is yielding new assays and biomarkers which, if validated, will be essential to the rational selection of microbicide candidates for efficacy trials (10).

In fact, a number of animal and human studies have produced evidence that levels of proinflammatory cytokine interleukin (IL)-1, IL-6 and chemokines IL-8, monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 3α (MIP3α) in cervicovaginal lavage (CVL) positively correlate with histopathological and clinical signs of vaginal inflammation (2, 9, 13-16). Accordingly, these cytokines/chemokines have been defined as biomarkers of cervicovaginal inflammation. Similar to systemic or other local inflammation, cervicovaginal inflammation is characterized by infiltration of leukocytes. However, these molecules are not leukocyte-specific and may be blocked by endogenous antagonists (39). These properties complicate the interpretation of cytokine profiles and, hence, their use as biomarkers of cervicovaginal inflammation. Thus, additional biomarkers are needed to facilitate the assessment of HIV-1 transmission risk and evaluate mucosal safety of microbicides.

As early as 2004, Fichorova and her colleagues found that IL-1, IL-6 and IL-8 could predict mucosal toxicity of vaginal microbicidal compounds by using an improved rabbit irritation model (13). In 2007, they reported two soluble adhesion molecules, E-selectin and VCAM-1 as biomarkers of leukocyte traffic and activation in the vaginal mucosa (39). These studies demonstrated that a single dose or three consecutive doses of detergents such as N9, SDS and BZK could induce obvious cervicovaginal inflammation, and soluble adhesion molecules might be used as biomarkers of mucosal inflammation besides proinflammatory cytokines and chemokines. This is understandable with regard to the pivotal role of adhesion molecules played in the onset and progress of inflammation and inflammatory...
diseases (5, 23, 25), in which a well-defined and regulated multistep cascade and initial capture of leukocytes are primarily mediated by the selectin family which is composed of three members: L-, P-, and E-selectins (25). In the present study, we tried to identify novel biomarkers of cervicovaginal inflammation and focus on the selectin family for preclinical assessment of microbicide in a murine model. By using a cytokine antibody array, which can analyze multiple proteins simultaneously, we identified two adhesion molecules, L-selectin and P-selectin, which significantly increased when mucosal inflammation was triggered by N9. Further studies suggest that L-selectin and P-selectin correlated closely with the severity of cervicovaginal inflammation induced by different proinflammatory agents. The early response and long duration of increased level of soluble L- and P-selectin after mucosal inflammation occurred suggest that L- and P-selectin may be recruited as additional cervicovaginal inflammation biomarkers and used for preclinical safety evaluation of microbicides candidate for the prevention of HIV-1 and other sexually transmitted diseases.

MATERIALS AND METHODS

Reagents. In the study, a panel of compounds was selected. While nonoxynol-9 (N9), benzalkonium chloride (BZK) and sodium dodecyl sulfate (SDS) were selected as positive controls in models of vaginal irritations and inflammation as reported in previous works (2, 9, 13, 15, 16), carboxymethylcellulose (CMC, which is widely used as microbicide excipients and control) and tenofovir (TFV, a microbicide candidate, which is proven to be efficacious and safe (1)) were chosen as negative controls. N9 and TFV were purchased from LKT Laboratories, Inc. (USA) and Molekula Limited (United Kingdom) respectively. BZK, SDS and CMC were purchased from
Sigma-Aldrich (USA). All agents were diluted in phosphate buffered saline (PBS) to avoid the confounding effects of differing formulations.

**Murine model.** Female C57BL/6 mice (6-8 weeks old) were obtained from Beijing Laboratory Animal Research Center and housed under specific pathogen-free (SPF) conditions in the Animal Center of Wuhan Institute of Virology, Chinese Academy of Sciences. Animal studies were performed according to Regulations for the Administration of Affairs Concerning Experimental Animals in China (1988), and protocols were reviewed and approved by the Laboratory Animal Care and Use Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. Seven days before the experiment, the reproductive cycle was synchronized through subcutaneous injection with 0.1 ml of Depo-Provera (medroxyprogesterone acetate injectable suspension, Pfizer Pharmacia & Upjohn Company, USA), and diluted in lactated Ringer’s saline solution for a final concentration of 30 mg/ml per animal (9).

Depo-Provera-treated mice were anesthetized with a formulation of pentobarbital (80 to 85 mg/kg of body weight) and received an intravaginal inoculation (10 μl) of compounds tested. The mice were treated with the following compound: 40 mg/ml or other indicated concentrations when considered with dose-dependent effects, of N9 in PBS, 40 mg/ml SDS in PBS, 20 mg/ml BZK in PBS, 10mg/ml TFV and 40 mg/ml CMC in PBS. Untreated mice and mice treated with the PBS alone were used as controls to evaluate normal tissue morphology and inflammation status in the cervicovaginal mucosal surface.

In this study, two different protocols were designed for parallel assessment of proinflammatory cytokine and soluble selectins release. In the first protocol, mice received a single dose of 10 μl compound in PBS or PBS alone, and CVL was collected 4 h after dose administration or at indicated time points when considered
with time-course effects. In the second protocol, mice were dosed on three consecutive days, and CVL was collected at 24 h intervals, 4 h after each treatment and on two consecutive days after the last application. CVL was collected from groups of 5-10 mice at the indicated time points by three consecutive washes with 40 μl of PBS per wash delivered and recovered 5 consecutive times, usually a total volume of 100-110 microliters was recovered. CVL was then centrifuged (×400 g at 4°C for 10 min by Thermo Scientific Sorvall Legend Micro 17R with rotor of 75003424) to separate the soluble supernatant from cell debris and stored at -80°C until analyzed. After lavage collection, mice from each treatment group were then immediately sacrificed, and the cervix and vagina were excised for histological analysis.

**Cytokine antibody array.** CVL was analyzed with cytokine antibody array by using RayBio Mouse Cytokine Antibody Array III (RayBiotech, Inc., Norcross, GA), according to the manufacturer’s instructions. Signal intensities were quantified directly with a chemiluminescence imaging system (FluorChem HD2, Alpha Innotech, USA) and analyzed with its software. Spots were digitized into pixel densities which were exported into Excel for analysis. For control group (PBS treatment), the pixel density (PD) of each protein was determined as the mean of duplicate spots. And then the fold change of each protein was calculated as $\frac{PD_{N9}}{PD_{control}}$.

**Flow cytometry assay.** Cells obtained from CVL were resuspended at a concentration of 1-2×10^6 cells/100 μl and blocked with Fc block (anti-CD16/ 32, eBioscience, 0.5 μg per test) for 15 min at 4°C and then incubated with fluorochrome-conjugated antibodies against mouse CD45 (0.03 μg per test), F4/80 (0.25 μg per test), and Gr1 (0.125 μg per test) (all from eBioscience) for 30 min at 4°C. Dead cells were excluded by 7AAD (BD Pharmingen, 0.5 μg per test) staining.
Samples were washed three times with 1% FBS in PBS and were resuspended in 300 μl of PBS containing 2% paraformaldehyde. Samples were assessed with FACSCalibur (Becton Dickinson, Heidelberg, Germany) and gated according to SSC, FSC and excitation spectrum. Leukocytes, neutrophils and macrophages were identified as CD45⁺, CD45⁺Gr1⁺ and CD45⁺F4/80⁺ cells, respectively (16). The counts were derived from the total viable cell count by the following formula: total viable cell count × % leukocytes (or macrophages or neutrophils (from differential staining)) = total number of leukocytes (or macrophages or neutrophils).

**Histological analysis.** Groups of 5-10 mice were treated intravaginally with tested compounds (or PBS for controls) and sacrificed at indicated times immediately before dissecting out the vagina. After macroscopic observations were recorded, each vagina was fixed in 5 ml 10% neutral-buffered formalin. The vaginas were embedded, sectioned horizontally, stained with hematoxylin and eosin (H&E), examined by bright-field microscopy, and evaluated blindly for epithelial cell disruption, leukocyte infiltration, edema and congestion. The histopathological scores were assigned by a semi-quantitative system which was based on Eckstein’s scoring system (11) and employed in our previous work (27). The detail of the scoring system was shown in Table S1 in the supplemental information.

**Determination of soluble factors by Enzyme-Linked Immunosorbent Assay.** Soluble factors in mice CVL was measured by ELISAs, which were run on diluted mouse CVL: 1:3 for MCP-1, IL-6, E-selectin and P-selectin, respectively, and 1:20 for L-selectin. Mouse MCP-1 and IL-6 kits were obtained from BD Biosciences (USA) and soluble E/L/P selectin ELISA kits were bought from Boster Biological Technology, Ltd. (China). Cytokine concentrations were determined using the calibration procedure and cytokine reference standards supplied with the ELISA kits.
There was no cross-reactivity between the different cytokines.

**Statistical analysis.** Except time-course effects (a single dose and three consecutive doses) were done with Two-Way analysis of variance (ANOVA), all other data analysis was performed with One-Way ANOVA. When the P value was significant at the 5% level, further pair-wise comparisons were made between the experimental group and control conditions using Dunnett’s test. Statistical analyses were carried out with Instat GraphPad software, version 5.0. Data are represented as mean ± standard deviation (SD) for individual mice of one representative experiment or as mean ± standard error (SE) of several experiments. The Pearson r (linear regression) analysis was used to assess correlation between the soluble factors and histopathology scores. A P value less than 0.05 was considered to be significant.

**RESULTS**

**Identification of novel protein molecules related to cervicovaginal inflammation by antibody array.** To identify novel biomarkers of cervicovaginal inflammation by antibody array, we first needed to determine the time point of lavage collection when proinflammatory molecules peaked. As Alt and Galen (2, 16) reported previously, MCP-1 was obviously increased in CVL by N9 treatment. Therefore, we first analyzed the kinetics of MCP-1. MCP-1 peaked at 4 hr (p<0.001) and then declined sharply at later time points (Fig. 1). Therefore, we collected CVL 4 hr after treatment with N9 and PBS (as negative control) to determine cytokine induction in the cervicovaginal environment by using a cytokine antibody array. In the N9-treated mice, thirteen proteins were significantly increased compared with PBS control (Figure 2): one binding protein, IGFBP-6 (insulin-like growth factor binding protein 6, 1.58-fold); two cytokines, including IL-6 (6.46-fold) and IL-12 p40/70...
(1.61-fold); three adhesion molecules: L-selectin (1.54-fold), P-selectin (1.49-fold) and VCAM-1 (vascular cell adhesion molecule 1, 1.98-fold); five chemokines: MCP-1 (3.4-fold), PF4 (Platelet factor 4, 1.47-fold), RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted, 1.58-fold), TARC (thymus and activation regulated chemokine, 1.29-fold), TCA-3 (T-cell activation-3, 1.45-fold) and two receptors: sTNFR I (Soluble Tumor Necrosis Factor Receptor, 1.25-fold) and sTNFR II (1.68-fold). Among these proteins, the most strikingly increased were cytokine IL-6 (6.46-fold) and chemokine MCP-1 (3.4-fold), both proinflammatory cytokines. Moreover, most of these proteins have been previously studied and showed increases with the introduction of proinflammatory compounds. Most interestingly, we also found that three adhesion molecules, including L-selectin, P-selectin and VCAM-1, exhibited significant increase after N9 treatment. In previous studies of soluble biomarkers for microbicidal safety, most attention was focused on proinflammatory cytokines and chemokines, while little attention was given to soluble adhesion molecules (2, 9, 13, 15, 16, 39). Actually, it is well documented that adhesion molecules play a pivotal role in the onset and progress of inflammation and inflammatory diseases (5, 23, 25). Inflammation usually consists of a well-defined and regulated multistep cascade; in fact, the initial capture of leukocytes is primarily mediated by the selectin family which is composed of three members: L-, P-, and E-selectins. Our results also suggest that L- and P-selectins may be involved in the cervicovaginal inflammation process. Thus, we focused our subsequent studies on the potential association of these soluble selectins and proinflammatory compound-induced cervicovaginal inflammation.

Time course and duration of the soluble L- and P-selectin level correlated
better than MCP-1 and IL-6 with those of toxic effects after a single application of N9. We next analyzed the time course of histopathology after vaginal application of a single dose of 40 mg/ml N9. At all time points, the morphological observations of the stained vaginal tissue sections revealed that the vaginal epithelium was a single-column layer, which is consistent with medroxyprogesterone treatment. The vaginal mucosa was intact in the PBS-treated mice with some leukocytes randomly spread throughout the tissue. However, N9 caused rapid exfoliation of epithelial cells and infiltration of leukocytes. Changes were detectable as early as 2 hr, and cumulative scores are statistically significant during 24 hr. The epithelial damage rose to a peak at about 4 hr and began to slowly regenerate after 12 hr of treatment, recovering almost to the control level by 48 hr. Leukocyte infiltration exhibited a similar increase, but resolved much earlier (at 8 hr). Edema was not seen in PBS-treated mice and only seen in some tissues at 4 and 6 hr in N9-treated animals. Conversely, dilation could be seen in all tissues, but it was more obvious in tissues from N9-treated mice (Table 1).

To verify the histological changes and further elucidate the quality and quantity of infiltrated leukocytes, leukocytes entering the vaginal tract in CVL were collected and analyzed by flow cytometric analysis. At all time points shown in Figure 3A, more than 95% of the leukocytes present in CVL of PBS-treated mice were neutrophils. In contrast, the percent of neutrophils of N9-treated mice was approximately 96% at 2 hr and then decreased following influx of macrophages. Leukocyte and neutrophil counts were variable among individual mice, and mean counts were not significantly different between N9-treated and control mice at any time point during this experiment (Figure 3A). Macrophage count exhibited a significant increase at the 12-hr time point after N9-treatment. The percent of
macrophages started to rise at 2 hr post-N9 treatment, reached a peak at 12 hr, then returned to the control baseline by 48 hr Because the percent of macrophages displayed a clear trend during the cervicovaginal inflammation process, we next used the macrophage percent as an index of leukocyte infiltration.

As shown in Fig. 3B and Fig. 1, proinflammatory cytokine IL-6 and chemokine MCP-1 in CVL increased only transiently, with a peak at the 2- and 4-hr time points respectively, followed by a sharp decline and immediate return to baseline values at the later time points. There was no significant increase of soluble E-selectin detected at any time point. Strikingly, L-selectin and P-selectin were rapidly increased at the 2- and 4-hr time point, respectively, and peaked at the corresponding later time point and persisting at significantly increased levels over 12 hr. It is noted that the significant release of soluble L-selectin and P-selectin was contemporaneous with tissue damage (total score), which started at 2 hr, peaked at 4 hr, and persisted for 12 hr with the most severe damage (Table 1). Therefore, the time course and duration of the soluble L-selectin and P-selectin level correlated better than MCP-1 and IL-6 with those of the tissue damage.

Soluble L-selectin and P-selectin were more sensitive than MCP-1 and IL-6 in determining mild degree of inflammation induced by low concentration of N9 treatment. To compare the sensitivity of soluble selectins with proinflammatory cytokine IL-6 and chemokine MCP-1 in determining cervicovaginal inflammation, we tested different degrees of inflammation by intravaginal application of various concentrations of N9 (40, 8, 1.6 and 0.32 mg/ml in PBS) or PBS only (0 mg/ml). Four hr after the treatment, significant tissue damage was found in mice treated with N9 at 1.6 mg/ml or higher concentration (Fig. 4A). Flow cytometric analysis demonstrated
that significant macrophage infiltration occurred in mice treated with N9 at 8 mg/ml or higher concentration (Fig. 4B).

Compared to zero dose (PBS only), a dose of 40 mg/ml N9 was sufficient to induce significant increases of MCP-1 and IL-6, while 8 mg/ml N9 could increase mild, but not significant, expression of MCP-1 and IL-6 (Fig. 4C, D). However, the 8 mg/ml N9 dose induced significant increase of L- and P-selectin (Fig. 4E, F) which correlated closely with the obvious tissue damages induced by respective dosage (Fig. 4A). These data demonstrated that L- and P-selectin can predict cervicovaginal inflammation and tissue damage more sensitively than MCP-1 or IL-6, especially for determining mild degree of cervicovaginal inflammation. No tested concentration of N9 exposure induced any significant increase of E-selectin (Fig. 4G).

L- and P-selectin were increased in animals treated with several detergent-based proinflammatory compounds. We wondered whether the increase of selectins in cervicovaginal inflammation is unique to N9 only or if it could be generalized to other proinflammatory compounds. Furthermore, it is intriguing to know the correlation of selectins and TFV, the only proved efficacious and safe microbicide candidate at present. To address these questions, we tested two additional proinflammatory compounds SDS and BZK, together with an excipient CMC, a microbicide candidate TFV, which were expected as noninflammatory besides PBS blank control. TFV and BZK were tested at concentrations of 10 mg/ml, 20 mg/ml, diluted in PBS respectively; N9, SDS, and CMC were tested at 40 mg/ml concentration, since this concentration has inflammatory consequences and increased susceptibility to HSV in a mouse model (9).

The histopathological examination of cervicovaginal tissue performed 4 hr after
a single compound application revealed that the vaginal mucosa was intact in the
PBS-, CMC- and TFV-treated mice with some leukocytes and dilated blood vessels
randomly spread throughout the tissue. The tissue from N9-, SDS- and BZK-treated
mice, however, showed various degrees of epithelial damage, leukocyte infiltration,
edema and dilation (Fig. 5A). BZK ranked first in epithelial damage with edema,
while N9 ranked first in leukocyte infiltration. All three detergents completely
disrupted the epithelial lining, while BZK damage extended to the lamina propria.
Multiple intra- and submucosal leukocyte infiltrates were found in the N9-treated
mice; however, leukocyte infiltration was also seen, albeit in a low degree, in BZK-
and SDS-treated mice. Dilated blood vessels were more obvious in tissues from N9-,
SDS- and BZK-treated animals than controls (Fig. 5A). All histopathology findings
were in accordance with previous reports (12, 39).

The results of flow cytometric analysis of cells collected from mice vaginal
lumen by lavages demonstrated that the percentage of macrophages remained
relatively unchanged after application of CMC and TFV, but increased significantly
after treatment with N9, SDS and BZK (Fig. 5B), compared with PBS control.

Four hrs after a single application, when compared with PBS alone, the excipient
CMC and the microbicide candidate TFV did not significantly increase any soluble
factor. On the contrary, all detergents tested here produced a significant increase in
two or more soluble factors. MCP-1 and IL-6 were only increased in one or two
detergents, and E-selectin remained unchanged in all detergents. However, L-selectin
and P-selectin were significantly increased in all detergents tested (Fig. 5C). As
Fichorova and her associates found that IL-1 and IL-8 could predict mucosal toxicity
of vaginal microbicidal (13), we also checked the level of IL-1β and KC, and found
that IL-1β was only increased by N9 treatment, and KC remained unchanged in all
Furthermore, as shown in Figure 5D, the Pearson’s correlation analysis between these five soluble factors and the total scores obtained in 30 test mice 4 hr after a single-dose administration found 1) a significant correlation between CVL L-selectin, P-selectin and IL-6 levels and the total score ($r=0.664, p<0.0001$; $r=0.665, p<0.0001$; and $r=0.523, p=0.003$, respectively); but 2) no significant correlation between MCP-1 ($r=0.257, p=0.171$) or E-selectin ($r=0.092, p=0.627$) and the total score. Hence, soluble L-selectin and P-selectin correlated better with the tissue damage.

In parallel, three consecutive compound applications with a 48-h post-treatment follow-up period showed a similar pattern of proinflammatory cytokines and soluble selectins (Fig. 6). All detergents tested here induced significant increase of L-selectin and P-selectin at 4 h after each application, and BZK also induced a delayed increase of these two molecules. N9, SDS and BZK also induced significant increase of E-selectin at some time points. On the other hand, MCP-1 and IL-6 were only significantly induced by N9 and BZK at a few time points and not triggered by SDS at all.

**DISCUSSION**

This study is the first to systematically characterize the soluble selectin levels in vaginal fluids during cervicovaginal inflammation induced by irritating compounds in a murine model. In addition, this is the first report to compare the levels of soluble selectins and proinflammatory cytokine IL-6 and chemokine MCP-1 in the prediction of cervicovaginal inflammation.

In progestine-treated C57BL/6 mice, we observed that the baseline CVL level of P-selectin was low and that the levels of L-selectin and E-selectin were moderate. A single dose or three consecutive daily doses of proinflammatory agents (N9, SDS or
BZK) promoted the significant increase of L-selectin and P-selectin, but E-selectin only in some occasions. Furthermore, compared with MCP-1 and IL-6, the predefined biomarkers of cervicovaginal inflammation, L-selectin and P-selectin had prolonged significant increase. They were also more sensitive, broad-spectrum indications of mucosal inflammation induced by different compounds, with better positive correlation to the time course and severity of inflammatory tissue damage. Thus, our study suggests that L-selectin and P-selectin should be novel biomarkers of cervicovaginal inflammation in addition to the proinflammatory chemokine MCP-1 and cytokine IL-6, improving the prediction of the proinflammatory side effects of other microbicide candidates for vaginal application.

The human cervicovaginal mucosa is the primary target of HIV-1 infection during male to female transmission. Mounting evidence indicates that cervicovaginal inflammation caused by sexually transmitted infections, bacterial vaginosis (BV) and some vaginal products, including microbicides, might increase HIV-1 infection risk (19, 22, 35). However, the mechanism underlying such phenomenon is not well known, and a validated biomarker of cervicovaginal inflammation is lacking. It has been suggested that epithelial damage or activation in the mucosa leads to IL-1β secretion and transactivation of NF-κB, resulting in release of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (IL-8, IL-10, and MIP 3α). The resulting influx of neutrophils and HIV-1 target cells can lead to subsequent HIV-1 infection and virus shedding (10). Because concentrations of these proinflammatory cytokines and chemokines in vaginal secretions correlate with epithelial damage, these cytokines/chemokines have been defined as biomarkers of cervicovaginal inflammation. However, these molecules reflect early events in the inflammatory cascade that may be blocked by endogenous antagonists that have pleiotropic or
opposing functions. These properties complicate the interpretation of cytokine profiles and their use as biomarkers of vaginal product safety. Therefore, markers specific for leukocyte infiltration, the focus of inflammation, would be a useful addition to current tools for monitoring cervicovaginal mucosal inflammation (10, 39).

Selectins mediate rolling, the first step of leukocyte infiltration, which is essential for both the development of an appropriate inflammatory response to injury or infection and the debilitating sequence of events leading to inflammatory diseases (20). The selectins are a calcium-dependent, type I transmembrane glycoprotein family of adhesion molecules. Three selectins exist, including E-selectin, P-selectin, and L-selectin, named for the cell type in which they were originally identified (endothelium, platelet, and leukocyte). All three selectins share a similar structure containing an N-terminal lectin-like domain, an epidermal growth factor-like domain, a variable number of consensus repeats (CRs), a single-transmembrane domain, and a short cytoplasmic tail (20). The most important ligand for selectins is the glycoprotein, P-Selectin Glycoprotein Ligand-1 (PSGL-1), which is present as a homodimer on leukocytes and can bind to the three selectins (25). Selectins are involved in leukocyte recruitment and in acute and chronic inflammation processes, including post-ischemic inflammation in muscle, kidney, heart, and skin inflammation, atherosclerosis, glomerulonephritis and lupus erythematosus (24). Soluble forms of selectins, which most likely arise by shedding or cleavage from the cell membrane, have been identified in serum and inflamed synovial fluid (4, 33, 43). Although considerable evidence has indicated that elevated serum concentration of soluble selectins is present in patients with different inflammatory diseases and correlate with disease duration and severity (5, 17, 36), only one previous study has evaluated the association between soluble E-selectin in cervicovaginal secretions and mucosal
inflammation (39).

In 2007, Trifonova and co-workers reported that soluble E-selectin was expressed in low, but detectable, levels in blank rabbits and was raised by BZK application. Similarly, our results showed that soluble E-selectin is also raised by three daily doses of detergents at some time points. However, there were also some differences existed: the baseline level of E-selectin in mice was moderate. The most rational explanation for this discrepancy is the difference in animal models studied: a rabbit model was used in Trifonova’s study and a murine model was used in ours (39). And the progestin treatment may be also a reason. In our study, to minimize the influence of reproductive cycle, the mice were pretreated with progestin 7 days before study, a treatment that produces a diestrous-like state, in which the epithelium is mucified columnar, and the lumen contains mucus and desquamated cells (9).

Consistent with previous studies (32, 37), our results showed that neutrophils distributed in the lumen and mucosal tissue of vagina from progestin-treated normal mice, but macrophages were rarely found. After surfactant treatment, neutrophils did not significantly change, but the percentage of macrophages was raised significantly. It is reported that every one of the three selectins is involved in neutrophil recruitment to skin and mucosal membranes, but P- and E-selectin are especially important. P- and L-selectin play key roles in monocyte infiltration, but the role of E-selectin is less significant (24, 34, 44). Therefore, it is reasonable that there should be a moderate level of E-selectin following a naturally occurring population of neutrophils in normal mice. Now that the percentage of neutrophils did not change significantly in cervicovaginal inflammation; accordingly, E-selectin had no significant change. However, P- and L-selectin rose significantly, followed by a large influx of macrophages, which was evidenced by P- and L-selectin association with the
increased percent of macrophages. Macrophages are important HIV-1 cell targets in vivo; therefore, the large influx of macrophages resulting from microbicide-induced mucosal inflammation would increase the chance of HIV-1 infection. This phenomenon is worth further study.

As shown in Figure 5, the increased levels of L-selectin and P-selectin in mice CVL were associated with the presence of an increased number of leukocytes and dilated blood vessels in tissue sections. Migrating leukocytes become the source of soluble L-selectin released into vaginal secretions. Besides activated endothelium and platelets, infiltrating macrophages may be another source of P-selectin because the molecule can also be expressed on macrophages (26, 38). To show some light on the origin of soluble L-selectin and P-selectin, we checked the surface expression of L- and P-selectin by flow cytometric analysis. As shown in Fig. S2 in the supplemental information, there were low but well-detectable levels of P-selectin expressed on vaginal leukocytes and the subpopulations, neutrophil and macrophage and the expression was down-regulated by N9 treatment. However, L-selectin was expressed at moderate level and there was no significant change by N9 treatment. These data gave us some clues that leukocytes and the subpopulation could be the origin of soluble L- and P-selectin.

Since the sole role of selectins is mediating leukocyte rolling and since soluble selectins arise from infiltrating leukocytes and activated endothelium, we reasoned that the significant increase of these molecules in cervicovaginal fluid could be a good additional predictor of cervicovaginal inflammation.

Until now, the rabbit vaginal irritation (RVI) model focused on histopathological findings is the only recommended animal model by the Food and Drug Administration of American to assess the toxicity of vaginal products. This model was improved by
Fichorova and her colleagues thereafter (13, 39). The refined model was expanded to include soluble markers of inflammation and vascular activation. Besides the classical RVI model, mouse model is also used in preclinical mucosal safety assessment for microbicide candidates. Despite the limitation that mice are not naturally susceptible to HIV, extensive experience with murine models in immunological study combined with low cost and the availability of a wide array of reagents suggested the feasibility for mouse model in the mucosal safety study of microbicides. A few studies examined the mucosal inflammatory response of intravaginal administrated N9 and SDS in mouse model and found exfoliation of epithelia, infiltration of neutrophils and macrophages into the genital lumen and tissue, increases in cytokines and chemokines and activation of the transcription factors NF-κB (7-9, 16, 32). Catalone and colleagues found that the cervix was the site of N9 and C31G-associated damage and inflammation, while the vaginal epithelium was resistant to the damaging effects of these surfactants (7, 8). However, our study found that the cervix (data not shown) and the vaginal epithelium are both sensitive to N9 irritation. In fact, by pretreatment with progestin, except the orifice of vagina is covered with the stratified squamous epithelium, the other parts are just covered with single column ones. Accordingly, the former is more resistant and the latter is more sensitive to irritation of N9 and other proinflammatory agents. Our study also agreed with other two previous studies (9, 16). Despite of some discrepancy, our study and Catalone’s study also had some agreements: N9 associated damage peaked around 2-4 hr post-application and was resolved by 24-48 hr post-exposure. The present study and the previous studies all showed that murine model are useful models of mucosal safety assessment of microbicide candidates. As progestins have distinct effects on the cervicovaginal epithelial immune function and therefore results in the depopovera treated mouse
model have to be interpreted with caution. The progestin treatment may be also a reason for some discrepancies between the mouse model study and other studies in rabbits and primates. Pre-treatment with progestin (Depo-Provera) markedly eliminate the influence of reproductive cycle and enhance the sensitivity to damage, but this cannot be practiced in humans. In non-human primate (NHP) or clinical trials, we can record carefully the menstrual cycle of NHP or women and analyzed the data collected grouped by menstrual cycle.

Clinical development of candidate microbicides is expensive and time-consuming; consequently, it is critical to ensure that products be safe and economically viable before moving from the preclinical to the clinical phase. Unfortunately, the current preclinical process is imperfect, and efforts are underway to develop new safety biomarkers and efficacy models (10). This study proposes novel soluble noninvasive markers of cervicovaginal inflammation in a murine model by comparison with the previously described biomarkers MCP and IL-6. Though only the epithelial damage proinflammatory compounds including N9, SDS and BZK are used as positive control in mouse model and L-selectin and P-selectin may not be suitable markers for other compounds that are toxic through other mechanisms, the results from this study support the use of soluble L-selectin and P-selectin as additional biomarkers of cervicovaginal inflammation in mouse model and warrant further assessment in humans. Moreover, the soluble level of selectins in serum tended to be greater in HIV-1-infected patients, and positive correlation with virus load and AIDS disease progression was evidenced by several previous studies (6, 21, 31). Therefore, further studies should examine their level in cervicovaginal secretions of healthy controls and HIV-1-infected patients to compare differences and evaluate the association of soluble levels of selectins and HIV-1 infection. Nonetheless, this study
has laid the foundation for the use of soluble L-selectin and P-selectin to monitor mucosal safety of any microbicide targeted for prevention of HIV-1 and other sexually transmitted infections, and additional measurements of these soluble molecules may enhance the efficiency of further efforts to develop successful novel microbicides to counter HIV-1 infection.

ACKNOWLEDGEMENTS

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Figure legends

**Figure 1.** MCP-1 in CVL peaks at the 4-hr time point after a single application of N9.

Mice were collected for CVL and sacrificed at indicated time points following a single application of 40mg/ml N9 in PBS or PBS alone. The untreated mice were regarded as baseline, i.e., 0 hour time point. 5 mice were set for each time point of each treatment. MCP-1 was quantified by ELISA in the supernatant of CVL collected at the indicated time point after a single 10 μl application of 40mg/ml N9. MCP-1 in CVL peaked at the 4-hr time point, followed by a sharp decline and return to baseline values at later time points. Experiments were repeated two times and showed similar results. Data are represented as mean ± SD for individual mice of one representative experiment. ***, p<0.001.**

**Figure 2.** Cytokine profile of murine CVL by N9 treatment.

Mice were treated intravaginally with a single 10 μl dose of 40mg/ml N9 in PBS or PBS alone for 4 hr, and CVL was harvested as described in Materials and Methods. The supernatant of CVL was subjected to RayBio mouse cytokine antibody array III, as described in Materials and Methods. Reactive protein spots were visualized by enhanced chemiluminescence detection. (A) Template showing the location of cytokine antibodies spotted in duplicate onto the RayBio Mouse Cytokine Array III. (B) Representative photographs of cytokine arrays using CVL from N9- or PBS (as control)-treated mice. Colored circles mark differentially expressed cytokines: purple, IL-6; red, MCP-1; blue, L-selectin; green, P-selectin; and brown, VCAM-1. CVL of thirteen mice per group were pooled for each experiment, and the cytokine array was repeated three times and showed similar results.. (C) The fold change of significantly increased protein by N9 treatment compared with PBS control. Data are represented as mean ± SD for duplicate spots.
of one experiment. POS, positive; NEG, negative.

**Figure 3.** Soluble selectins show distinct time course comparing with proinflammatory cytokine by application of N9.

Mice were collected for CVL and sacrificed at indicated time points following a single application of 40mg/ml N9 in PBS or PBS alone. And the untreated mice were regarded as baseline, i.e., 0 hour time point. 5 mice were set for each time point of each treatment. Supernatant was separated from cell debris by centrifugation. (A) Recruitment of inflammatory cells into the vaginal lumen as determined by flow cytometric analysis. Leukocytes of each group were harvested by lavage, pooled and stained for expression of CD45, Gr1 and F4/80. Gates for leukocytes, neutrophils or macrophages were set on CD45/FSC, CD45/Gr1 or CD45/F4/80 dot plots to identify the desired subsets. Leukocytes, neutrophils and macrophages were identified as CD45+, CD45+Gr1+ and CD45+F4/80+ cells, respectively. Data are represented as the mean ± SE of two experiments. (B) Soluble factors were quantified by ELISA in supernatant of CVL. Proinflammatory cytokine IL-6 in CVL increased only transiently. For E-selectin, there was no difference between the N9 treatment group and controls at all time points. Notable exceptions were soluble L-selectin and P-selectin, which were rapidly increased and persisted at significantly increased levels over twelve hr. Experiments are repeated two times and showed similar results. Data are represented as mean ± SD for individual mice of one representative experiment. *, p<0.05; **, p<0.01; ***, p<0.001: N9 treatment group versus corresponding PBS control.

**Figure 4.** L- and P-selectin are more sensitive than IL-6 and MCP-1 in indication of
In three independent experiments, a group of ten mice were treated intravaginally with a single dose of serial five-fold dilution of 40 mg/ml N9 up to 0.32 mg/ml. CVL was collected at 4 hr after application. Supernatant was separated from cell debris by centrifugation and was detected for soluble factors by ELISA. Cells of each group were pooled and analyzed for macrophages by flow cytometric analysis. Mice were sacrificed immediately after lavage collection, and vaginas were dissected for histology analysis. (A) Cumulative (total) scores are shown for one representative experiment with 5 animals per group. (B) Macrophages as a percent of leukocytes of 3 independent experiments, as determined by flow cytometric analysis. (C-G) soluble factors of CVL: C) MCP-1; D) IL-6; E) L-selectin; F) P-selectin; G) E-selectin. *, p<0.05; **, p<0.01; ***, p<0.001: various concentrations (except 0) of N9 treatment group versus zero concentration (PBS control). (A and C-G) Data are represented as mean ± SD for individual mice of one experiment. (B) Data are represented as mean ± SE value of 3 independent experiments.

Figure 5. L- and P-selectin correlate closely with the severity of mucosal inflammation induced by N9, SDS and BZK.

A group of ten mice were treated intravaginally with a single dose of 40 mg/ml CMC, 10mg/ml TFV, 40 mg/ml N9, 40 mg/ml SDS and 20 mg/ml BZK in PBS or PBS alone. CVL was collected at 4 hr after application. Supernatant was separated from cell debris by centrifugation and was detected for soluble factors by ELISA. Cells of 3-5 mice from each group were pooled and analyzed for macrophage by flow cytometric analysis. Mice were sacrificed immediately after lavage collection, and vaginas were dissected for histology analysis. (A) Representative
photograph comparing histological findings in full-thickness paraffin-embedded abdominal vaginal sections from mice treated with a single application of CMC, N9, SDS and BZK in PBS or PBS alone. H&E staining; magnification ×400. (B) Percent of macrophages present in leukocytes was determined by flow cytometric analysis of macrophages prepared from CVL. Left and middle: Density plot of isotype control and N9 treatment for one representative experiment to show how macrophage percent was determined. Right: data are represented as mean ± SE value of three replicate tests of one experiment. (C) Soluble factors: MCP-1, IL-6, L-selectin, P-selectin and E-selectin. Increases in L-selectin and P-selectin were detected in CVL from mice treated with all detergents tested; increases in IL-6 and MCP-1 were detected only after treatment with N9 or BZK. Data are represented as mean ± SD for individual mice of one experiment. (D) Pearson’s correlation analysis of cumulative (total) mice vaginal irritation scores and MCP-1, IL-6, L-selectin, P-selectin and E-selectin for 30 animals. Dotted lines represent the 95% confidence intervals. The correlation coefficients (r) and p values are also shown. (B-C) *, p<0.05; **, p<0.01; ***, p<0.001: detergent group versus PBS control.

**Figure 6.** Increase in soluble selectins, MCP-1 and IL-6 levels by consecutive doses of N9, SDS and BZK. Mice were treated intravaginally with three consecutive doses of 40 mg/ml CMC, 10mg/ml TFV, 40 mg/ml N9, 40 mg/ml SDS and 20 mg/ml BZK in PBS or PBS alone. CVL was collected at 4 hr after each application and 28 and 52 hr after the last application. Supernatant was separated from cell debris by centrifugation and was detected for soluble factors by ELISA. Each dose administration is marked by a black arrow. Responses of proinflammatory chemokine MCP-1 and IL-6 in mice CVLs were shown in (A) and (B) respectively. Responses of soluble L-selectin, P-selectin and E-selectin in mice CVLs were shown in (C), (D) and (E) respectively. Results represent mean ± SD from
five mice for one experiment. *, p<0.05; **, p<0.01; ***, p<0.001: proinflammatory compound treatment group versus PBS control at each time point.
# Figures and Tables

## Table 1. Time points of composite mice histopathology scores obtained after a single administration of 40mg/ml N9 in PBS or PBS alone (as control)

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Mice were sacrificed at the indicated time points following a single application of 40mg/ml N9 in PBS or PBS alone. And immediately the vaginal tissues were taken and processed for histopathological examination which was evaluated blindly for epithelial cell disruption, leukocyte infiltration, edema and congestion. Untreated mice were utilized as baseline controls, i.e., the zero time point, to document the normal tissue architecture and inflammation status in the vaginal mucosa. The histopathological scores were assigned by a semi-quantitative system which was based on Eckstein’s scoring system (11) and employed in our previous work (27). The detail of the scoring system was shown in Table S1 in the supplemental information. Data are represented as mean ± SD obtained from five mice per group of one experiment.

- a) p<0.05; b) p<0.01; c) p<0.001, as compared with that of corresponding control group (Two-Way ANOVA, followed by Bonferroni post-tests).
### Table A

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### Image B

**PBS**

**N9**

### Image C

![Bar graph showing fold change](https://via.placeholder.com/150)

- **Cytokine**
- **Chemokine**
- **Adhesion Molecule**
- **Receptor**
- **Binding Protein**

Legend:

- IL-6
- IL-12 p40/70
- MCP-1
- PF4
- RANTES
- TARC
- TCA-3
- L-selectin
- P-selectin
- VCAM-1
- sTNFR I
- sTNFR II
- IGFBP6