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Measurement of mucosal biomarkers in a phase 1 trial of intravaginal 3% SPL 7013 gel (VivaGel®) to assess expanded safety

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Abstract

Objective—The aim of this study was to examine the effect of the 3% SPL 7013 gel (VivaGel®) on mucosal immune markers hypothesized to be associated with HIV-1 acquisition.

Design—Phase 1, placebo-controlled, randomized, double-blind clinical trial was performed in 54 young women in the U.S. and Kenya. Participants used carbopol gel with and without (placebo) SPL 7013 twice daily over 14 days. Cervical specimens were collected for cytokines, chemokines, T-cells, and dendritic cells at day (D) 0, 7, 14, and 21. A negative binomial regression model was used to assess differences between study arms.

Results—Several mucosal immune parameters were increased in the VivaGel® arm compared to placebo. For cytokines: D 7, IL-6 (p=0.05); D 14, IFN- γ (p=0.03), IL-2 (p=0.04), IL-5 (p=0.003) and IL-10 (p=0.001) were increased. On D 7, CD8+/CD69+ T-cells tended to be increased (p<0.08); limiting analysis to visits without blood or bacterial vaginosis, these findings were stronger: at D7, CD8+/CD69+ T-cells were increased in the VivaGel® arm (p<0.005), as were CD4+/CD69+ cells (p=0.001) and CD4+/CCR5+ T-cells (p=0.01). The changes described for D7 and 14 were no longer seen at D21.

Conclusion—Markers associated with inflammation and epithelial damage were reversibly elevated in the VivaGel® arm compared to the placebo arm after 7–14 days of twice daily product use.

Keywords

Vaginal microbicide; mucosal immune markers

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Introduction

The HIV pandemic has motivated the research community to develop self-applied vaginal microbicides. An ideal vaginal microbicide would prevent HIV and other sexually transmitted infections, be safe, acceptable and affordable. However, defining such products as safe has proven difficult. Several microbicides that were determined to be safe in phase 1/2 trials based on clinical criteria were subsequently found to increase the risk of HIV acquisition in phase 2 and 3 clinical trials. One example is the trial examining the spermicide nonoxynol-9 (N-9) which was thought to be a safe product until one trial showed N-9 resulted in an increased risk of acquiring HIV.¹

Possible means by which microbicides may increase HIV risk include physical breach of epithelial integrity, mucosal immune cell activation (i.e., enhancing the number of target cells and/or their expression of HIV-binding receptors), and immune inhibition.² All these modes of injury are associated with the complex expression of immune markers including cytokines, chemokines, antimicrobial peptides and local influx of activated B and T cells. Consequently, researchers have gone back to pre-clinical *in vitro* and animal studies with previously unsuccessful microbicides in order to better define biomarkers which may reflect the observed harm associated with their use. Studies of N-9 found mucosal elevation of numerous cytokines and chemokines including macrophage inflammatory protein (MIP)-2, interleukin (IL)-1 α , IL-1 β , IL-6 and IL-8 and tumor necrosis factor (TNF).³⁻⁵ These cytokines have been associated with cell damage and the local recruitment of immune cells, including those expressing HIV-adhesion molecules such as CCR5+ and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN). Fichorova *et al*⁴ also showed that N-9 was associated with diminished genital levels of secretory leukocyte protease inhibitor (SLPI), a factor that is thought to be important in host defense against bacterial pathogens. These results suggest that phase 1 microbicide trials should incorporate the measurement of these mucosal biomarkers in the genital tract as a more sensitive measure of harm to screen investigational products.

We recently published results from a phase 1 placebo-controlled, randomized, double blinded clinical trial of SPL 7013 (VivaGel[®]) applied twice daily over 14 days.⁶ VivaGel[®] is a unique dendrimer compound that showed efficacy in preventing simian immunodeficiency virus (SIV) infection in non-human primates and herpes simplex virus (HSV)-2 in the guinea pig model, and was shown to be safe in a phase 1 trial using a single daily dose over seven days. Our results demonstrated no grade 3 or 4 adverse events (AE) and good tolerability.⁶ However, participants in the VivaGel[®] arm in comparison to the placebo arm had a greater number of grade 1 and 2 genitourinary adverse events (AE) and superficial colposcopic findings. Similar findings were reported in another phase I trial of VivaGel[®].⁷ One of the secondary objectives of this project was to examine the effect of VivaGel[®] on immune markers that may be associated with HIV acquisition among participants enrolled in the phase 1 trial, including cytokines, chemokines, activated T-cells and dendritic cells expressing HIV-adhesion molecules and SLPI. This manuscript reports these findings. In addition, we examine the correlation of these biomarkers with visual evidence of epithelial disruption in the lower genital tract.

Methods

This was a Phase 1, placebo-controlled, randomized, double blind clinical trial in sexually-abstinent young women, conducted at the Clinical Research Center at the University of California, San Francisco (UCSF), USA, and the Research Care and Training Program unit of the Center for Microbiology Research at the Kenya Medical Research Institute (KEMRI) in Kisumu, Kenya. The women used 3.5 grams carbopol gel with and without (placebo) SPL

7013 (VivaGel®) twice daily over 14 days. The study protocol and informed consent forms were approved by the Committee on Human Research at UCSF and the National Ethical Review Committee at KEMRI. Safety oversight was provided by Independent Safety Monitors and a Safety Monitoring Committee. This trial is registered at www.ClinicalTrials.gov (NCT00331032).

Enrolment and design were described in detail previously.⁶ Briefly, women were eligible if they were 18–24 years of age, sexually experienced, not pregnant within 3 months, known to have regular menstrual cycles, and had not started a new long-acting contraception (e.g. depomedroxyprogesterone) within the past 3 months. Women were also excluded if at screening they had a positive test for urinary tract infection (UTI), HIV or HSV-2 antibodies, syphilis, vaginal candidiasis, symptomatic bacterial vaginosis (BV) using Amsel's criteria, vaginal Nugent score ≥ 7 ⁸, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, or *Chlamydia trachomatis*, and abnormal cervical cytology. Women who had epithelia disruptions of the anogenital tract were also excluded.

Women were asked to be sexually abstinent one week prior to enrollment and throughout the 21 days of study participation. The enrollment visit was scheduled to fall within 5–14 days after the first day of the next menses. A pelvic examination including a visual examination, colposcopy, vaginal pH, vaginal sample for semen exposure and vaginal wet mount were performed. The following were collected for immune parameters: a cervicovaginal lavage (CVL) was performed with 5ml of phosphate buffered saline (PBS) and reaspirated for cytokine analysis; a cervical cytobrush was placed into 5ml of cellular transport medium (RPMI with 10% FBS [Sigma]) for cell analysis by flow cytometry. After collection, cytobrush specimens were stored at 4°C and transported to the laboratory on ice within 2 hours. Women were asked to return for follow-up examinations and collection of immune parameters on days 7, 14 and 21. Women were instructed not to insert study gel at least 6 hours prior to any of the examinations. Details of examinations were previously reported and included colposcopic examinations and testing for vaginal and cervical infections.⁶

Lab methods

Analysis of cell types and surface markers

Personnel at each site were trained to process samples by a single person and all samples were received blinded to randomization status. Upon arrival at each site's laboratory, cytobrush samples for cell analysis were vortexed vigorously to dissociate cell clumps and mucus before removing the cytobrush, and the cell suspension was filtered through a 100 micron nylon cell strainer (Becton Dickinson). The resulting suspension was centrifuged at 15rpm for 10 minutes, the supernatant decanted and the cell pellet reconstituted into 1ml of freezing medium (heat inactivated fetal bovine serum [Sigma] with 10% DMSO). The cryovial was placed into a freezing chamber that had been pre-chilled overnight to –20°C with isopropanol (Mister Frosty, Nalgene Fischer Scientific) and cooled overnight to –80°C prior to transfer into a –150°C freezer. Samples were transported to a central laboratory in a liquid nitrogen dry shipper (MVE Vapour Shipper) for analysis. Cells were thawed, washed and divided into two equal aliquots for staining with a panel of dendritic cell (DC) or T-cell markers. Cells in the T-cell aliquot were stained with CD69-FITC, CCR5-PE, CD4-PerCP and CD3-APC (BD Pharmingen, San Jose, California, USA); cells in the DC aliquot were stained with CD11a-FITC (Imgenex San Diego, California, USA), CD11c-PE, CD14-PerCP and DC-SIGN-APC (eBioscience, San Diego, California, USA) according to manufacturer's instructions. Appropriate isotype control antibodies were used in parallel. Samples were processed using FACSCalibur (Becton-Dickinson Immunocytometry Systems). Cell

numbers enumerated by flow cytometry (in the T-cell or DC aliquot) were multiplied by two to determine “cells per cytobrush.”

Analysis of cytokines and SLPI

Cervicovaginal lavage specimens were cryopreserved at -80°C within 2 hours of collection, and transported to a central laboratory in a liquid nitrogen dry shipper (MVE Vapour Shipper) for analysis by laboratory staff blinded to randomization status. Cell-free supernatants of the CVL specimens were tested for cytokines as previously described⁹ using the LINCOplex High-Sensitivity Human Cytokine Immunoassay Kit (Millipore, Billerica, MA) and the Luminex-100 platform (Luminex, Austin, TX) in duplicate according to the manufacturer’s instructions. The following cytokines and chemokines were included: Granulocyte macrophage colony-stimulating factor (GMCSF), ILs -1β , -2 , -4 , -5 , -6 , -8 , -10 , -12 , and -13 , TNF, and interferon gamma (IFN- γ). SLPI levels were measured by ELISA (Quantikine Human SLPI kit, R&D Systems), according to the manufacturers’ instructions.

Statistical analysis

We treated all the outcome variables (i.e. cytokines, and T-cell and DC markers) as counts, and used negative binomial regression to examine differences in outcomes between VivaGel[®] and placebo groups. Alternate models fitted using Poisson regression provided similar results, but did not fit the data as well. Because this analysis was a secondary objective of the primary phase I trial and considered exploratory, the regression analyses described below did not adjust for multiple testing for different cytokines.

For baseline (day 0) comparison between VivaGel[®] and placebo arms, we fitted all the models with arm as the independent variable, and values of cytokines, T-cell and DC markers as dependent variables. Because the cells expressing the early activation marker depend on the initial number of DC or T-cell counts, we included the total cell counts (in logarithm form, such as CD4, CD14) as offsets in regression models. Resulting coefficients can be interpreted as the log relative proportion of cytokine counts (with denominators given by the total cell counts) compared between treatment groups.

For longitudinal observations of cytokine levels we fitted additional negative binomial models including time on study grouped as day 7, 14, and 21 (and using day 0 as reference), and evaluated between group differences via an interaction between time and treatment group. The exponentiated coefficients of the interaction terms can be interpreted as time-specific relative treatment comparisons similar to those described above. Within individual correlation in outcomes were accounted for using robust standard errors estimated using generalized estimating equation (GEE) methods.

For purposes of data presentation, we present for each day the percent of samples in each arm that were above the 75% quartile. Quartiles were calculated by combining the two arms for each day. The median, 25th and 75th percentiles for the cytokines and cell markers are shown in Table 1. Since 80% of the samples had undetectable levels for IL-5, the median, 25th and 90% percentile are presented for IL-5. We found two outliers in analysis of IL-10 at Day 14 in placebo group, which substantially affected the result of negative binomial model. Therefore, we removed them from the model. Although outliers were found for other cytokines, removing them did not alter any of the findings and consequently were not removed.

The similar modeling approaches were adopted to examine the effect of adverse events (AE) on outcome variables indicated above, except that we included the variable AE as the sole

independent variable in the models. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

Since the immune markers were obtained from the cervicovaginal lavage specimen (soluble markers) and cervix (cell markers), AEs included in this analysis were limited to evidence of epithelial disruption (ED) in the vagina and cervix. This included vaginitis, cervicitis, abrasions, petechiae, lacerations and erythema. Vulvar and perianal findings and reported GU symptoms (e.g. pain, discharge) were not included for the purposes of this analysis. Women who were non-compliant with study product at any of the visits, defined as using < 80% of expected doses, were excluded from all analyses. Since blood from menstrual bleeding, trauma from the speculum or presence of bacterial vaginosis could alter cytokines, we also performed a sensitivity analysis excluding those visits if blood was noted on examination or BV was diagnosed. Three participant visits had bacterial vaginosis diagnosed and 10 had blood visualized in the vaginal vault. Data is not shown for these results, only p-values.

Results

In summary, a total of 54 women were enrolled. ⁶ Of the 54 women, 35 women were enrolled in the VivaGel[®] arm and 19 in the placebo arm. The mean age (20.8 years) was similar in both arms. In addition, lifetime number of sexual partners and number of sexual partners in the last 3 months was also similar. ⁶ The San Francisco site was predominantly white and the Kisumu arm was 100% black African.

Most (83%) participants, including 30 (86%) in the VivaGel[®] arm and 15 (79%) in the placebo arm, reported taking at least 80% of doses ($p = 0.52$). Thirty-three of 205 (16%) total follow-up visits had a cervical and/or vaginal finding suggestive of epithelial disruption (ED)—of these, 25 (19%) were in the VivaGel[®] arm and 8 (11%) in the placebo arm, $p = 0.17$.

Cytokine/chemokine results

No differences were seen at day 0 between the VivaGel[®] and placebo arms for GMCSF, IFN- γ , ILs -1 β , -2, -4, -5, -6, -8, -10, -12, and -13, and TNF. Using negative binomial regressions models, we examined differences between arms at day 7, 14, and 21 using day 0 as the comparison day. At day 7, higher levels of IL-6 were found in the VivaGel[®] arm. These findings are summarized in figure showing the percent of samples from each day in each arm that was in the top quartile. For IL-5, percent of samples that were 90th percentile or greater is shown. On day 21 (1 week after discontinuation of study product), no differences were found except that IL-4 ($p=0.007$) and GMCSF ($p=0.002$) were lower in the VivaGel[®] arm. No differences were seen for SLPI on any of the days between study arms.

When we excluded the visits with blood or bacterial vaginosis in the sensitivity analysis, the findings were similar. At day 7, there was a trend for IL-6 as it was higher in the VivaGel[®] arm ($p = 0.1$). At day 14, similar changes were also found in the VivaGel[®] arm in comparison to the placebo arm: higher levels of IFN- γ ($p = 0.03$), IL-2 ($p = 0.04$), IL-5 ($p = 0.003$) and IL-10 ($p = 0.008$). At day 21, IL-4 ($p = 0.16$) and GMCSF ($p = 0.02$) were similarly lower in the VivaGel[®] arm in comparison to the placebo arm.

T-cells and DC cell markers

We found no differences between VivaGel[®] and placebo for any of the T-cell and DC markers at day 0 except for total number of CD8+ T-cells which were greater in the VivaGel[®] arm ($p=0.02$). At day 7, VivaGel[®] had a trend for greater number of CD8+ / CD69+ T-cells ($p < 0.08$) (figure 1). On day 14, no differences were seen for any of the T-

cell or DC cell markers. At day 21, CD11+/DC-SIGN+ cells were higher in the VivaGel® arm ($p = 0.05$).

In the sensitivity analysis with the visits with blood or bacterial vaginosis excluded, the findings remained similar. At day 7, CD8+/CD69+ T-cells were significantly greater in the VivaGel® arm ($p < 0.005$). In addition, CD4+/CD69+ cells ($p = 0.001$) and CD4+/CCR5+ T-cells ($p = 0.01$) were higher in the VivaGel® arm. At day 21, there was a similar trend for greater numbers of CD11+/DC-SIGN+ cells in the VivaGel® arm ($p = 0.14$).

Correlation of vaginal and cervical epithelial disruption (ED) with immune markers

The presence of cervical and vaginal ED was associated with the elevations of several cytokines including IL-4 ($p = 0.001$), IL-10 ($p = 0.007$), IL-13 ($p < 0.001$), IL-1 β ($p = 0.007$), and TNF ($p = 0.012$). A trend was seen for IFN- γ ($p = 0.1$). The total number of CD4+ and CD8+ T-cells were greater on cytobrushes collected from participants with ED ($p=0.02$ and $p=0.009$, respectively) and decreases were noted for CD4+/CD69+ T-cells ($p = 0.07$), CD4+/CCR5+ T-cells ($p = 0.012$), and CD4+/CD69+/CCR5+ T-cells ($p = 0.02$). In order to see if the biomarkers added information beyond visible EDs, we performed another sensitivity analysis by excluding all visits with ED. Although the sample size was diminished significantly, the p -values for all the biomarkers were similar (data not shown) except for IL-6 at day 7 which demonstrated a similar trend toward higher levels in the VivaGel® arm, but the p -value was no longer significant ($p=0.3$).

Discussion

The ideal microbicide should retain its *in vitro* activity against HIV and other STIs *in vivo* without disrupting the integrity of the epithelial barrier, inducing inflammation or adversely interfering with innate immunity. In this phase 1 study, several findings associated with VivaGel® were of concern. These included increases in genital cytokines and T-cell subsets that were associated with clinical findings of epithelial disruption, and that have been associated with an increased risk for HIV acquisition in women.

Epithelial damage can result in the release of several cytokines and chemokines including IL-1 β , IL-6 and TNF, IL-8, and IL-10.^{5, 10} In accordance with this, visible epithelia damage in the vagina and/or cervix observed in our cohort was associated with increases in IL-1 β , IL-10 and TNF. We also saw elevations in the Th2 cytokines, IL-4 and IL-13, which have also been implicated in tissue damage.^{11, 12} In comparison, the findings associated with VivaGel® compared to placebo also showed a mixed picture of cytokine alterations which included greater levels of IFN- γ .

In looking at the trend of cytokine expression across the 4 visit dates, the difference between the VivaGel® arm and placebo arm at day 7 and 14 was in part due to the fact that the women in the placebo arm had lower levels on these days than seen at baseline. Because of the protocol design which enrolled women between days 5 to 14 of their menstrual cycle, the Day 7 visit normally occurred mid-cycle and the Day 14 visit occurred during the luteal phase. Several studies have shown that there are significant differences in cytokine levels seen during the menstrual cycle¹³, with consistent changes seen in IL-6 and IL-8 levels. Shrier et al¹⁴ found mid-cycle dips whereas Al-Harthi et al¹⁵ found much lower levels of IL-6 and IL-8 in the luteal phase compared to the follicular phase. Not all studies showed such contrasts during the menstrual cycle.¹⁵⁻¹⁷ The absence of mid-cycle and luteal cytokine dips in the VivaGel® arm may be interpreted as altering the normal immune cycle, and hence detrimental. The impact of such variations on HIV susceptibility *in vivo* is not clear.

Epithelial damage also can lead to lymphocyte extravasation which, in the case of HIV, may enhance infection. Myer et al.¹⁸ showed that visible evidence of cervical epithelial disruption increased the risk of HIV acquisition. After epithelial injury, CD69+/CD8+ and CCR5+/CD4+ T-cells would be normally found in the epithelial mucosa as part of the host defense.

Unfortunately, CD4+ T-cells are also target cells for HIV.¹⁹ Interestingly, the number of CD4+ and CD8+ T-cells in our study were increased in those with evidence of epithelial damage but activated CD4+ T-cells (CD69+ and CCR5+) were decreased for reasons not understood. Studies with N-9 demonstrate the product was associated with an influx of CD4+ T cells. Although we did not observe increases in the sheer number of CD4+ or CD8+ T-cells with VivaGel[®], we did observe greater numbers of activated CD8+ T-cells in the VivaGel[®] arm at Day 7 compared to placebo; however, this difference was not sustained at Day 14.

Except for N-9, few other microbicide studies have incorporated immune markers. Bollen *et al.*²⁰ examined the effects of Carrageen, a relatively inert substance without an apparent effect on HIV acquisition. In their study, Carrageen, as well as the placebo gel caused a decrease in the levels of IL-8 and IL-6, suggesting that the gels may have caused some inhibition in the assays. Decreases in IL-8 levels have been observed with the use of several microbicides, including cellulose sulfate, Pro2000 and BufferGel.^{21–23} Pro2000 and BufferGel were also associated with lower levels of IL-1 β and SLPI. Since the placebo in our trial was the same substance (Carbopol) used as the vehicle for VivaGel[®], the dampening observed in the placebo arm for many of the cytokines was not likely due to inhibition by the vehicle itself.

One of the strengths of this investigation was that participants were all enrolled and subsequently followed at similar times of their menstrual cycle. A second strength of the study is that women were screened for numerous STIs and were excluded if any of the tests were positive. Third, the close observation of the women allowed us to control for possible factors such as visible blood and bacterial vaginosis which may also influence immune markers. Lastly, women agreed to abstain from sexual intercourse one week prior to enrollment and throughout follow-up and very few women had objective evidence of semen exposure.⁶

Limitations include our small sample size which was consistent with most phase 1 trials. Also, the clinical interpretation of these findings is limited since there are no human trials that have yet shown that these immune markers define absolute risks for female HIV acquisition, although results from CAPRISA-004 are forthcoming.²⁴ Rather, the evidence for risk of HIV acquisition related to altered innate and adaptive immunity in the female genital tract are based primarily on *in vitro* and animal studies.^{3–5} In addition, the study involved two different populations. The impact of race and ethnicity could not be studied in this small sample size. However, in a previous publication⁹, we found significant immune differences between the two groups at baseline prior to product exposure. Future studies should include the potential impact of race and ethnicity on the association of microbicide products on mucosal immune changes in the genital tract.

In summary, in this phase 1 clinical trial, markers associated with epithelial damage were elevated in the VivaGel[®] arm compared to the placebo arm after 7–14 days of twice daily product use. Most of the changes were reversed 7 days after discontinuation of the product. These findings need to be taken with a certain degree of caution due to their exploratory nature and small sample size. However, these findings and the observed increase in adverse

events reported in the two phase 1 trials should be taken into consideration in planning any future clinical trials.

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Figure 1.

Graphs demonstrate the percent of samples that were in the top quartile (> 75%) for VivaGel[®] and Placebo at days 0, 7, 14 and 21 for cytokines and cell markers that were significant at either day 7 or day 14. The arrows demonstrate the significant time point generated by the binomial regression analysis. Quartile cut off values are shown in Table 1. P values are shown in the text. For IL-5, the percent of samples that were 90th percentile or greater is given.

Table 1
Median and interquartile (25th and 75th percentile)/ distribution for cytokines and cell markers

	Day 0	7	14	21
IFN- γ	0.19 (0.06 ² , 0.67)	0.06 ² (0.06 ² , 0.30)	0.06 ² (0.06 ² , 0.53)	0.06 ² (0.06 ² , 0.72)
IL-2	0.06 (0.06 ² , 0.27)	0.06 (0.06 ² , 0.23)	0.06 (0.06 ² , 0.18)	0.06 (0.06 ² , 0.25)
IL-4	0.38 (0.06 ² , 1.64)	0.06 (0.06 ² , .43)	0.24 (0.06 ² , 0.74)	0.53 (0.06 ² , 3.69)
IL-5	0.06 (0.06 ² , 0.13)	0.06 (0.06 ² , 0.08)	0.06 (0.06 ² , 0.41)	0.06 (0.06 ² , 0.15)
IL-6	10.2 (3.04, 26.3)	5.84 (1.42, 14.52)	13.72 (2.81, 91.04)	10.12 (3.58, 82.79)
IL-10	0.35 (0.06 ² , 1.36)	0.094 (0.06 ² , 0.36)	0.12 (0.06 ² , 0.93)	0.56 (0.16, 8.55)
GMCSF	0.06 (0.06 ² , 0.54)	0.06 (0.06 ² , 0.12)	0.06 (0.06 ² , 0.24)	0.06 ² (0.17, 2.75)
CD8+/CD69+	28 (8, 49)	12 (5, 46)	24 (12, 74)	14.5 (4, 39)
CD11+/DCS+	141 (89, 351)	199 (60, 908)	206 (79, 506)	204 (68, 294)

¹ all cytokine values reflect the value in pg/ml and cell markers reflect number of cells per cytobrush at the median with the 25th and 75th percentile values in parenthesis. Because 80% of the values for IL-5 were undetectable, the range given in parenthesis reflects the 25th and 90th percentile.

² .06 is value assigned to below detection