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Preclinical safety assessment of Griffithsin-based vaginal microbicides.

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PRECLINICAL SAFETY ASSESSMENT OF GRIFFITHSIN-BASED VAGINAL MICROBICIDES

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B.S., University of California, Davis, 1999

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A Thesis Approved on

July 31, 2013

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ABSTRACT

SAFETY AND IMMUNOGENICITY OF THE NATURAL LECTIN GRIFFFITHSIN

IN MICE

Amanda Lasnik

July 31, 2013

Griffithsin (GRFT) is a protein derived from the red alga *Griffithsia sp.* off the coast of New Zealand. It binds and inactivates number of enveloped viruses including HIV-1. For a product to be developed as a microbicide, it must be safe and effective after repeated use. For initial *in vivo* safety testing we exposed mice repeatedly to various vaginal products and found that increased concentration of mouse serum albumin (MSA) in the vaginal lumen is predictive of epithelial damage. Repeated intravaginal administration of GRFT did not result in increased amounts of MSA, but the known epithelial disrupters nonoxynol-9 and benzylkonium chloride caused increased concentrations of MSA that were detectable by ELISA and western blot. We also evaluated the immunogenicity of GRFT following repeated exposure and found that intravaginal administration resulted in higher serum IgG antibody titers, but not local IgA or IgG levels, than did subcutaneous administration. The consequences of the systemic immune response are unknown and require further investigation.

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CHAPTER 1

INTRODUCTION

HIV Biology

Human Immunodeficiency Virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). It is in the lentivirus family of retroviruses. The genome consists of two copies of single-stranded RNA comprising nine open reading frames which encode 15 proteins. The *gag* gene encodes a polyprotein which is further processed into the viral structural proteins including the matrix (p17), capsid (p24) and nucleocapsid (p6 and p7) proteins. The enzymes required for the viral life cycle - reverse transcriptase, protease, and integrase - are encoded by the *pol* gene. The *env* gene codes for the precursor glycoprotein gp160 which is processed to give rise to the viral envelope glycoproteins gp120 and gp41. The regulatory genes *tat* and *rev* and the accessory genes *nef*, *vpu, vif,* and *vpr* encode their eponymous proteins.

The gp120/gp41 spike plays an important role in attachment and entry of the virus to the host cell, and is therefore a major focus of current research. The

precursor protein gp160 is synthesized in the endoplasmic reticulum, where it oligomerizes to form a trimer. The trimer is transported through the trans-Golgi network, where it is glycosylated primarily with high-mannose glycans and cleaved by furin to produce the surface (gp120) and transmembrane (gp41) glycoproteins. These glycoproteins associate with each other in a non-covalent manner. The initial step in HIV infection is the association of gp120 with the host cell's CD4 receptor. This step is followed by interaction of gp120 with the chemokine co-receptor CCR5 or CXCR4. CCR5 is the major co-receptor initially associated with sexually transmitted HIV; advanced AIDS is marked by a shift in the preferential binding of gp120 to CXCR4. The interaction of gp120 and the co-receptor results in a conformational change which exposes gp41. This mediates fusion of the virus to the cell membrane and results in viral entry. The entry process is outlined in **Figure 1-1**.
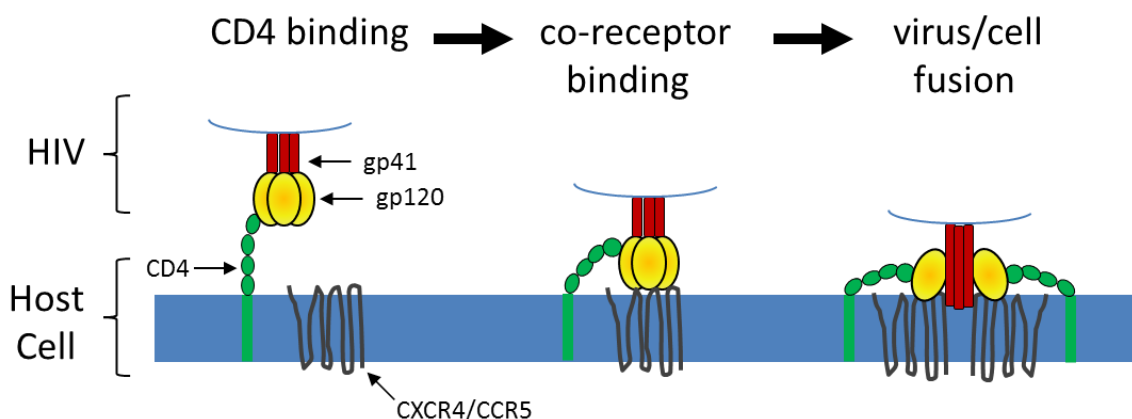
Figure 1-1. The three main steps in HIV entry into the cell.

Prevention of HIV by Microbicides

There are currently 33.3 million people infected with HIV-1, with 2.6 million new infections each year (UNAIDS Global Report, 2010). A vaccine to prevent HIV-1 infection remains a priority for researchers, but developing a vaccine against this virus is complicated by a number of factors. HIV-1 exhibits tremendous genetic diversity due to its error-prone reverse transcriptase which introduces approximately 1 error for every round of replication. This makes it difficult for the immune system to target the virus effectively. Additionally, HIV-1 targets the immune system, which compromises the host's ability to adequately fight infection. HIV-1 inserts copies of its reverse-transcribed DNA into cells, creating a reservoir of the virus and thus necessitating life-long treatment with drugs that will keep the HIV viral load low. While a clinical trial demonstrated moderate success (31% efficacy) for a vaccine, further research is necessary [1]. Until an efficacious vaccine is developed, other methods of prevention are required.

Women are more likely to contract HIV from an infected heterosexual partner than vice versa, especially in regions of high HIV incidence. Current strategies for risk reduction – abstinence, monogamy, and consistent condom use – are often not feasible for women to negotiate, and male circumcision does not protect women from HIV. Additionally, products which prevent sexually transmitted infections but maintain fertility are of particular interest. Therefore a woman-controlled alternative to the male condom must be considered to slow the spread of HIV.

Microbicides are products that can be applied vaginally or rectally prior to sexual intercourse to prevent the transmission of HIV-1 or other sexually transmitted infections. They may be broadly classified in one of three categories: (1.) non-specific HIV inhibitors; (2.) agents that prevent viral replication; and (3.) entry inhibitors that prevent binding of the virus to cellular receptors. Early efforts in the microbicide field used products that had non-specific mechanisms of action against HIV. Though *in vitro* assays showed promise, clinical trials with the spermicidal detergent Nonoxynol-9 (N-9) demonstrated an increase in the risk of infection with repeated use [2,3,4]. C31G (Savvy) also showed good in vitro bactericidal and virucidal properties; however two large clinical trials were halted due to lower than expected seroconversion rates in both arms of the trial population. Additional data analysis indicated a non-significant trend towards harm in the C31G users compared to the placebo group [5]. Acidifying agents that lower the vaginal pH and inactivate HIV and other pH-sensitive pathogens comprise another group of non-specific HIV inhibitors. Although these agents are inexpensive and readily available, clinical studies proved them to be ineffective [6]. **Table 1-1** outlines some of the clinical trials to date. A comprehensive systematic review and meta-analysis all major clinical trials can be found in [7].

Product	Category	Clinical trial outcomes	Ref.
Cellulose Sulfate / Ushercell	Non-specific inhibitor (anionic polymer)	Two Phase III studies halted due to increased HIV incidence in one study	[8]
Pro 2000	Non-specific inhibitor (anionic polymer)	Phase III trial completed Product showed safety but not efficacy	[9]
BufferGel	Non-specific inhibitor (acidifier)	Phase II trial completed Initial results indicate it is not efficacious	[10]
N-9	Non-specific inhibitor (detergent)	Phase III trials completed Increased HIV incidence in experimental group	[2,3,4]
Carraguard	Non-specific inhibitor (anionic polymer)	Phase III trial completed Product showed safety but not efficacy	[11]
C31G / Savvy	Non-specific inhibitor (detergent)	Phase III study halted due to low HIV incidence in study population	[5]
Tenofovir	Viral replication inhibitor	Phase II trial completed 39% overall reduction in new HIV infections	[12]

Table 1-1. Selected microbicide clinical trial outcomes.

Agents that prevent viral replication represent a second class of microbicides. In June 2010 a report outlined the success of the CAPRISA 004 Phase II trial employing a gel formulation of 1% tenofovir, a nucleotide reverse transcriptase inhibitor [12].   The study showed an overall reduction in HIV acquisition of 39% and a reduction of 54% for those with high protocol adherence. Surprisingly, 1% tenofovir gel also protected against herpes simplex virus type 2 (HSV-2) infection, with an efficacy of 51% in this study (reviewed in [13]). HSV-2 is a well-established co-factor for HIV transmission and therefore the results of this study were encouraging. However a larger follow-up study (VOICE trial) showed no efficacy whatsoever and was halted due to futility. The

CAPRISA 004 trial used a protocol in which women were instructed to use the gel within 12 hours before sexual intercourse and again within 12 hours after sexual activity, while daily administration was used in the VOICE study. It's unknown whether a difference in protocol or a difference in adherence between the two trials resulted in disparate results, and further studies are pending. There are additional safety concerns regarding the use of antiretroviral drugs for prevention. In an earlier clinical trial, systemic absorption was detected in a majority of participants [14]. Absorption of low levels of this effective drug may select for viral resistance in those already infected, and further studies are required. Because of the highly mutable nature of HIV, microbicidal products will likely be formulated as a combination of two or more drugs with different mechanisms of action, such as tenofovir combined with an entry inhibitor. .

Agents that interfere with the interaction between HIV and the host may be further classified into two groups: those that bind to the virus, and those that bind to the receptors. Compounds that target cellular receptors are attractive candidates because they presumably will not cause escape mutants; however care must be taken to ensure that these agents do not interfere with receptor function. Agents in this class of compounds include monoclonal antibodies against CD4, CCR5 inhibitors, analogs of the chemokine RANTES, and inhibitors of C-type lectins such as DC-SIGN. The monoclonal antibody ibalizumab is specific for CD4 and prevents interaction with HIV, but does not block MHC class II molecules [15]. It is currently in clinical trials as an injectable therapeutic, but could be developed as a topical agent. Since CCR5 is the primary co-receptor

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for sexually transmitted HIV-1 and predominates during early stages of infection, compounds that sequester or block CCR5 at the mucosal surface may provide protection. However it remains to be seen if blocking CCR5 will result in an increase in infection caused by viruses that use CXCR4 to gain entry. Synthetic analogs of the chemokine RANTES, such as PSC-RANTES, also inhibit CCR5. PSC-RANTES provided complete protection against SHIV SF162 in macaques, although the dose required was relatively high at 1 mM [16]. Finally, several studies have implicated C-type lectins, especially DC-SIGN, as a major mechanism by which HIV is trapped by antigen presenting cells and presented to CD4+ cells [17,18,19]. Mannan, a mannose-rich oligosaccharide derived from yeast, was able to block DC uptake of HIV in vitro, but was ineffective in preventing HIV transmission in a macaque challenge with SHIV [20].

Compounds that bind to the HIV envelope and prevent cellular attachment are especially promising. The CD4-binding pocket of the viral envelope protein gp120 is highly conserved and small molecules or monoclonal antibodies that target this region are potential candidates. The broadly neutralizing human monoclonal antibody 2G12 is specific for the glycans on gp120. While it showed promise in early clinical trials in a cohort of patients infected with HIV-1, it also resulted in escape mutants in a majority of these participants [21,22]. Another target is the cellular fusion of the virion, which is mediated by the gp41 trimer. Enfuviritide is a synthetic peptide that binds gp41 and prevents viral fusion. Unfortunately, this peptide readily causes escape mutants when administered to

patients with HIV [23]; it is unknown if selection of mutants would be enhanced by topical administration.

While monoclonal antibodies and other recombinant proteins may provide good protection against HIV, the cost to produce them at a large scale could be prohibitively expensive. We recently described the production of an HIV microbicide, Griffithsin (GRFT), via a plant-based expression system employing a recombinant viral vector [24]. Because the upstream costs of plant manufacture are lower than traditional "closed" cell culture systems, the cost to manufacture GRFT per dose is similar to that of the male condom, a practical requirement for the use of microbicides.

Antiviral lectins as candidate microbicides

Many viruses, including HIV-1, are heavily glycosylated. The HIV-1 envelope protein gp120 is more than 50% glycan by mass. Approximately 40% of the N-linked glycosylation sites are occupied by the high-mannose type glycans, with the remainder of the sites occupied by complex-type glycans. HIV uses this glycan shield to evade the host immune system and also to facilitate infection of T cells via C-type lectins, such as DC-SIGN, present on dendritic cells. These glycans are attractive targets for HIV inhibition.

Many lectins are derived from natural sources such as plants, cyanobacteria, and marine algae, and most of these have potent HIV inhibitory activity. Several of these lectins are summarized in Table 2. GRFT, first

described in 2005, is a 12,770 Da protein derived from the red alga *Griffithsia sp.* off the coast of New Zealand [25]. It is the most potent HIV inhibitor described in the literature, with IC_{50} values in the low picomolar range. The crystal structure has been determined to be a domain-swapped homodimeric molecule [26]. GRFT has 3 mannose-binding sites in each monomeric unit, for a total of 6 high-mannose binding sites. It is hypothesized that these multiple binding sites crosslink the virus, thus accounting for GRFT's potency. A recent report outlining the ability of a single monomeric GRFT to bind two different nonamannoside molecules via all 3 carbohydrate binding sites strengthens this hypothesis [27].

Because GRFT is not as well characterized as other lectins, it is instructive to compare its features to a more well-known microbicidal candidate, cyanovirin-N (CV-N). CV-N was originally isolated from the cyanobacterium *Nostoc elliposporum* [28], and has recently been expressed in transgenic tobacco plants [29]. Expression of CV-N in transgenic tobacco circumvented the original problem of the bacterially-produced protein's low yield and aggregation. CV-N is an 11,000 Da protein with 2 glycan-binding domains. Early structural studies indicated that the protein existed as a monomer in solution and a domain-swapped dimer in crystal form [30,31]; however a recent evaluation by NMR shows no evidence of domain-swapping, and thus the sugar-binding properties of CV-N are due to multiple sites on the monomer [32].

Early pre-clinical efficacy studies with CV-N showed promise. CV-N inhibited infection of human cervical explants with HIV-1 [33]. It did not induce toxicity at the macroscopic or microscopic level in rectal epithelia of pigtailed

macaques treated daily for 7 days with a 1% gel, nor in a single intravaginal dose [34]. With either a 1% or 2% intrarectal treatment, 100% of macaques were protected from intrarectal challenge with SHIV89.6P. 7 of 8 animals in each group were protected from intravaginal challenge following treatment with 0.5%, 1% or 2% CV-N, while all animals in placebo groups were infected. However, although no apparent toxicity to human explants or to macaques was observed, CV-N, like many other lectins, showed pronounced mitogenic activity on lymphocytes [35]. GRFT, on the other hand, has no mitogenic activity [24].

One possible drawback for the use of lectins such as CV-N and GRFT is their potential, like all foreign proteins, to be immunogenic. It is not known how an immune response to these drugs will affect their potent antiviral properties. Thus, an extensive set of experiments must be completed to determine how immunogenic these candidates are and how this immunogenicity may affect their utility as microbicides.

CHAPTER 2

MOUSE SERUM ALBUMIN IS A SENSITIVE MARKER FOR EPITHELIAL DAMAGE IN A MURINE PRE-CLINICAL MODEL

Under normal circumstances, the human vaginal epithelium is an effective barrier to HIV infection, with HIV transmission events estimated at 1 in every 1000 incidents [36,37]. However, upon disruption of this natural barrier the rate of HIV-1 acquisition is drastically higher. The vaginal epithelia may be breached by inflammation and ulceration due to other sexually transmitted infections, disruption of the normal lactobacilli-dominated bacterial flora, or micro-trauma caused by sexual intercourse. Any of these occurrences may lead to HIV target cells infiltrating the site of epithelial disruption. It is therefore of utmost importance to ensure that any microbicidal product does not result in damage or inflammation of the vaginal epithelium.

The outcomes of several recent clinical trials have highlighted the shortcomings of the current preclinical safety evaluations of microbicidal products. Repeated use of the FDA-approved spermicide N-9 resulted in an increase in HIV acquisition [2,3,4]. More recently, a CONRAD-sponsored

efficacy trial of a cellulose-sulfate based microbicidal gel was halted as a precaution after a non-statistically significant trend towards harm in the experimental group was observed [3]. The preclinical safety assays used for these products failed to predict the trial outcomes.

The current Gold Standard toxicity test for FDA approval of topically-applied vaginal products is the Rabbit Vaginal Irritancy (RVI) assay, first described in 1969 [38]. This model involves daily administration of the test product for 10 days. The endpoints are anatomical and histopathological evaluation of the cervicovaginal mucosa. The tissue is evaluated by a pathologist in a total of four categories: 1.) ulcerations and erosions of the epithelial lining; 2.) thickness of the submucosal layer (edema); 3.) leukocyte infiltration of the submucosal layer; and 4.) erythema. Each sample is scored from 0 (normal) to 4 (severe) for each of the categories, and the total comprises a Mean Irritation Score (MIS). One insufficiency of this assay is that it was designed for identifying compounds that cause major irritation, and thus it lacks the sensitivity to detect minor irritants that could still lead to enhanced susceptibility to HIV acquisition. N-9 is perhaps the best example of this deficiency. With an MIS <8, N-9 is identified by the RVI as a "minor" irritant suitable for human use, however macroscopic evaluation shows evidence of increased leukocyte infiltration, edema, and vascular congestion, as well as disruption of the epithelial lining [39].

The RVI is increasingly viewed by those in the field as a tool for identifying the maximum tolerated dose of a product, and newly developed assays are used

to identify products which cause epithelial toxicity by means other than massive irritation. These pre-clinical models employ cell and organ culture, as well as animal models other than the RVI. Cell cultures are useful for evaluating drug effects on cell viability and pro-inflammatory cytokine release. By monitoring changes in the transepithelial electrical resistance (TER) of a dual chamber cell culture system, Mesquita *et al* identified epithelial toxicity of cellulose sulfate (CS) that was not predicted by the RVI or macaque challenge models [40]. The changes in TER after N-9 or CS exposure correlated with downregulation of junctional protein gene expression, which likely provides a route for HIV and other viruses to cross the epithelium. Bridging the gap between cell culture and clinical trials, tissue explants provide an invaluable tool for screening compounds using relevant human tissues. Reconstructed human vaginal-ectocervical epithelia are commercially available (MatTek) and can be used to evaluate the effects of topical microbicides on tissue viability, induction of cytokines and chemokines, and HIV susceptibility [41,42]. This model is more complex than traditional cell culture assays, but fails to recapitulate the interaction with the mucosal and systemic immune response of an *in vivo* model. A newly developed mouse model for preclinical testing of vaginal products has recently been described and is increasingly considered an important method for evaluating the safety of topical microbicides. This model employs transmission of herpes simplex virus-2 (HSV-2) as a surrogate marker for HIV transmission. HSV-2 is an important risk factor for acquisition of HIV-1, and thus this model represents an important advance for the microbicides field. Pathological changes in the

mouse vaginal epithelium similar to those seen in rabbits and humans were observed for both single [43] or repeated [44] doses of N-9. This model was also predictive of the risk associated with cellulose sulfate-based microbicides, which the RVI did not foresee [40].

Although advances are being made in the field of preclinical microbicide safety, models employing HSV-2 or changes in TER require specialized training and equipment. We hypothesized that detection of elevated levels of mouse serum albumin (MSA) in vaginal lavage samples of mice could provide a simple and relatively inexpensive method for screening candidate microbicides.

Movement of exogenous serum albumin across disrupted epithelium has been used in systems other than the genital tract to show transport of macromolecules and monitor loss of tight junctions. Fluorescently labeled bovine serum albumin was shown to accumulate in the basolateral layer of bronchial epithelial cells exposed to cigarette smoke *in vitro* [45]. Additionally, an *in vivo* model of inflammatory bowel disease demonstrated that efflux of labeled BSA into the intestinal lumen of mice following perfusion was associated with NFκB-mediated internalization of tight junction proteins [46]. The work presented in this chapter provides evidence that detection of endogenous serum albumin is a sensitive marker of epithelial disruption in the genital tract *in vivo*.

MATERIALS AND METHODS

Materials

The following over-the-counter vaginal products were purchased at Walgreen's pharmacy: KY Jelly® (McNeil-PPC), Conceptrol® 4% Nonoxynol-9 (Caldwell Consumer Health), Norforms® Deodorant Suppositories (C.B. Fleet), Monistat®, 2% Miconazole Nitrate Cream (McNeil-PPC). Pharmatex®, 1.2% Benzalkonium Chloride (BZK) (Innotech Laboratories) was purchased online from a pharmacy in the Czech Republic. Griffithsin was formulated in Carbopol gel at 0.1% (w/v) by Dr. Lisa Rohan's laboratory at the Magee Women's Research Institute at the University of Pittsburgh School of Pharmacy. Four blinded microbicidal gels (CO-JU10-21, -22, -23, -24) were provided by Dr. Gustavo Doncel's laboratory at CONRAD.

Animal Experiments

Groups of 5 BALB/c mice were injected subcutaneously with 3 mg Depo-Provera (Pfizer) 3 or 4 days prior to intravaginal treatments. Mice were treated intravaginally for 7 or 10 days with 40 µl of the products. Carbopol gel or sham treatment was used as a negative control.

Vaginal washes were collected one day after the last treatment by dispensing and aspirating 150 µl of 1X PBS containing protease inhibitor (G-Biosciences) 10 times, and then repeating for a total collection of 300 µl lavage fluid. The lavage samples were centrifuged at 800 rcf for 10 minutes, and the supernatant was stored at -20°C.

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Measurement of protein concentration in lavage fluid

Protein concentration of CVL was quantified by the Bicinchoninic Assay (BCA). BCA substrate was prepared per the manufacturer's instructions (Pierce). 20 µl of undiluted sample was incubated with 200 µl of BCA substrate in a 96-well microtiter plate for 30 minutes at 37°C. Plates were read on a BioTek Synergy HT plate reader at 562 nm.

SDS PAGE/western blot

Equal volumes of CVL samples were diluted in 2X Laemmli buffer and separated by SDS PAGE (18% Tris-HCl gels, Bio-Rad) at 200V and stained with SafeStain (Bio-Rad) according to the manufacturer's instructions. For detection by western blot, the samples in the gel were transferred to methanol-wetted polyvinylidene difluoride (PVDF) in transfer buffer (10 mM CAPS pH 10.5, 15% (v/v) methanol). The membrane was blocked in 5% non-fat dry milk (NFDM) in 1X PBS containing 0.05% Tween-20. Bands were detected using anti-MSA antibody (AbCam) at 1:1000, followed by anti-rabbit IgG-HRP secondary at 1:10,000. The membrane was incubated in ECL substrate (GE Healthcare) for 5 minutes and exposed to X-ray film.

Identification of proteins of interest by Mass Spectrometry

Bands in the Conceptrol group running at ~68 kD on SDS PAGE were analyzed by in-gel tryptic digestion and mass spectrometry. The gel was cut into small pieces and placed in an eppendorf tube. Approximately 2 volumes of

50mM NH_4HCO_3 was added and incubated at room temperature for 15 minutes.

1 gel volume of acetonitrile was added and incubated for 15 minutes. The

solvents were removed and the gel pieces were placed in a speedvac until dry.

The pieces were then swelled with 20 mM DTT in 0.1M NH_4CO_3 and incubated

for 45 minutes at $56^{\circ}C$. After removal of the DTT solution, the gel pieces were

incubated for 30 minutes in the dark with 2 volumes of 55 mM iodoacetamide in

0.1M NH_4CO_3. The iodoacetamide was removed and the gel washed twice with

5-10 volumes of 50 mM NH_4CO_3 for 15 minutes. Following a final wash of 3

volumes of NH_4CO_3, 4 gel volumes of acetonitrile was added and incubated for

15 minutes. The solvents were removed and the gel was dried in a speedvac.

The dried gel pieces were digested with 20 ng/ul modified trypsin (Promega) in

50 mM NH_4CO_3 at $37^{\circ}C$ for 4 hours. The samples were desalted with C18

ZipTip. MS/MS data was acquired by LTQ-Orbitrap XL (Thermo Scientific). A

database search was performed using Bioworks (Thermo Scientific).

Protein quantitation by Enzyme-Linked Immunosorbent Assay (ELISA)

Samples were diluted 25-fold in 1X PBS and 100 ul was plated in

microtiter plates (Nunc Maxisorp). Mouse serum albumin (Sigma) was diluted to

1 ug/mL and serially diluted 3-fold in 1X PBS to serve as a control. Plates were

incubated overnight at $4^{\circ}C$. After washing in 1X PBS-T, samples were blocked in

5% non-fat dry milk in wash buffer for 2 hours at room temperature. Plates were

washed and incubated in rabbit anti-MSA at a 1:5,000 dilution in 1X PBS for 1

hour. After washing, plates were incubated with horseradish peroxidase-

conjugated goat anti-rabbit IgG diluted 1:10,000 in 1X PBS. After a final wash, plates were developed with SureBlue Reserve TMB substrate (KPL) and development was stopped with addition of 1N H_2SO_4.

Total IgA and IgG levels were measured by double antibody sandwich ELISA. Microtiter plates were incubated overnight with unlabeled capture antibody (Southern Biotech). After blocking, vaginal wash samples or purified immunoglobulin were diluted in 1X PBS and incubated for 2 hours. HRP-conjugated secondary antibody against IgA or IgG was employed for detection. Plates were developed and absorbance was measured as for the MSA ELISAs.

The pro-inflammatory cytokines IL-1β and IL-6 were quantified by double antibody sandwich ELISA per the manufacturer's instructions (eBiosciences). Vaginal wash samples were diluted 5-fold in 1X PBS. All washes were performed on an Immunowash automated plate washer (BioRad). Plates were developed and absorbance was measured as for the MSA ELISAs.

Statistics

Graphpad Prism software version 5.04 was used for graphing results and statistical analysis. Data were analyzed by the Kruskal-Wallis test with Dunn's Multiple Comparison posttest. A p-value of < 0.05 was considered significant.

RESULTS

To identify MSA as a marker for epithelial disruption, CVL samples were diluted 2-fold in Laemmli buffer and separated by SDS PAGE. Following staining, a band was observed at approximately 68 kDa in all of the mice treated with Conceptrol®, but the band was faint or not present in the mice treated with KY Jelly® (**Figure 2-1**).

To positively identify the protein, the samples were analyzed by Mass Spectrometry. The CVL samples were separated by SDS PAGE and stained. Bands at the appropriate molecular weight were excised from the gel and an in-gel tryptic digest was performed. The digested protein was run on a LTQ-Orbitrap. A database search was performed using Bioworks Software, and the protein was identified as mouse serum albumin (**Figure 2-2**).

To evaluate the effect of over the counter (OTC) vaginal products, mice were treated daily for 10 days to determine if any commercially available products resulted in increased levels of MSA, IgA, IgG, IL-1β or IL-6 in the CVL. All commercially-available products were purchased at Walgreens, except for Pharmatex®, which was purchased online from a pharmacy in the Czech Republic.

CVL samples were analyzed for MSA levels by western blot and ELISA (**Figure 2-3**). By western blot, the sham treatment and Norforms had the lowest amount of MSA in the CVL. The CVL from mice treated with Pharmatex® clearly contained the highest level of MSA. The western blot results were in agreement

with the levels of MSA quantified by ELISA. By the Kruskal-Wallis test, mice

treated with either Conceptrol® and Pharmatex® had significantly more MSA in

the CVL than the mice in the sham treatment group (p = 0.001).

Total IgA and IgG levels were quantified by ELISA (**Figure 2-4**). Overall

levels of IgA were higher and more variable than for IgG. None of the test

groups had a statistically significant increase in IgA levels over untreated mice (p

= 0.1445). For total IgG, only mice treated with BZK had a markedly higher

concentration of this immunoglobulin in their CVL than any other mice (p =

0.0013). Neither IgA nor IgG predicted damage by N-9 treatment.

The pro-inflammatory cytokines IL-1B and IL-6 were also analyzed in the

CVL of mice treated with various vaginal products (**Figure 2-5**). IL-1β levels

were low overall, with KY Jelly producing the highest average concentration.

None of the treatment groups had a statistically significant increase of IL-1β over

the untreated group (p = 0.3045). IL-6 concentrations were highest in the N-9

treated animals, followed by BZK (p = 0.0241).

To determine if detection of MSA could predict damage resulting from

formulated microbicidal gels such as Cellulose Sulfate, a panel of four gels was

obtained from CONRAD. Mice were treated daily for 10 days. As in the previous

experiment, CVL samples were analyzed by western blot and ELISA (**Figure 2-
6**).

As for the experiment employing OTC products, the mice in this

experiment treated with Pharmatex® clearly had the most MSA in the CVL by

immunoblot analysis. One mouse in the KY Jelly® group and two mice in the

CO-JU10-24 groups had comparable levels of MSA to the mice treated with

Pharmatex[®]. The mice in the other three treatment groups (CO-JU10-21, -22,

and -23) exhibited similar MSA levels to sham-treated mice. The ELISA results

for the blinded groups were not in complete agreement with the western blot

data. By ELISA, the Pharmatex[®]-treated mice again contained a significantly

increased concentration of MSA in the CVL as compared to the sham treatment

group ($p = 0.0058$). However, in contrast to the western blots, the median

concentration of MSA in the CO-JU10-24 group was much lower than for CO-

JU10-23 (0.446 µg/mL vs. 3.207 µg/mL).
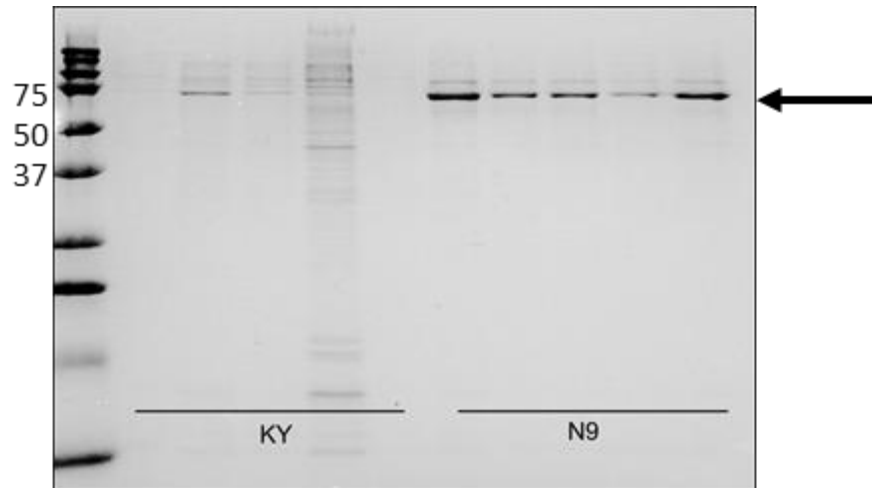
Figure 2-1. **SDS PAGE of cervicovaginal lavage samples.** Mice were treated

daily for 10 days with KY Jelly® or Conceptrol® containing nonoxynol-9 (N-9).

CVL samples were boiled with 2X loading dye and separated on an 18% gel.

Kaleidoscope molecular weight marker was used to approximate protein

molecular weights. Bands were visualized by staining in SafeStain. The arrow

indicates the band corresponding to mouse serum albumin (MSA), which is

approximately 68 kDa.

Number of Amino Acids: 608   Monoisotopic MW: 68648.7   pI: 5.69

Protein:

```
MKWVTFLLLL FVSGSAFSRG VFRREAHKSE IAHRYNDLGE QHFKGLVLIA
FSQYLQKCSY DEHAKLVQEV TDFAKTCVAD ESAANCDKSL HTLFGDKLCA
IPNLRENYGE LADCCTKQEP ERNECFLQHK DDNPSLPPFE RPEAEAMCTS
FKENPTTFMG HYLHEVARRH PYFYAPELLY YAEQYNEILT QCCAEADKES
CLTPKLDGVK EKALVSSVRQ RMKCSSMQKF GERAFKAWAV ARLSQTFPNA
DFAEITKLAT DLTKVNKECC HGDLLECADD RAELAKYMCE NQATISSKLQ
TCCDKPLLKK AHCLSEVEHD TMPADLPAIA ADFVEDQEVC KNYAEAKDVF
LGTFLYEYSR RHPDYSVSLL LRLAKKYEAT LEKCCAEANP PACYGTVLAE
FQPLVEEPKN LVKTNCDLYE KLGEYGFQNA ILVRYTQKAP QVSTPTLVEA
ARNLGRVGTK CCTLPEDQRL PCVEDYLSAI LNRVCLLHEK TPVSEHVTKC
CSGSLVERRP CFSALTVDET YVPKEFKAET FTFHSDICTL PEKEKQIKKQ
TALAELVKHK PKATAEQLKT VMDDFAQFLD TCCKAADKDT CFSTEGPNLV
TRCKDALA
```

Protein Coverage:

Sequence	MH+	% Mass	AA	% AA
YNDLGEQHFK	1250.58	1.82	35 - 44	1.64
LVQEVTDFAK	1149.62	1.67	66 - 75	1.64
SLHTLFGDK	1017.54	1.48	89 - 97	1.48
LCAIPNLR	899.51	1.31	98 - 105	1.32
ENYGELADCCTK	1345.54	1.96	106 - 117	1.97
NECFLQHK	1018.48	1.48	123 - 130	1.32
DDNPSLPPFERPEAEAMCTSFK	2481.11	3.61	131 - 152	3.62
ENPTTFMGHYLHEVAR	1901.90	2.77	153 - 168	2.63
ALVSSVR	731.44	1.07	213 - 219	1.15
LSQTFPNADFAEITK	1681.84	2.45	243 - 257	2.47
LATDLTK	761.44	1.11	258 - 264	1.15
ECCHGDLLECADDR	1578.60	2.30	268 - 281	2.30
YMCENQATISSK	1374.60	2.00	287 - 298	1.97
LQTCCDKPLLK	1261.66	1.84	299 - 309	1.81
AHCLSEVEHDTMPADLPAIAADFVEDQEVCK	3383.52	4.93	311 - 341	5.10
DVFLGTFLYEYSR	1609.79	2.34	348 - 360	2.14
RHPDYSVSLLLR	1455.81	2.12	361 - 372	1.97
HPDYSVSLLLR	1299.71	1.89	362 - 372	1.81
KYEATLEK	981.53	1.43	376 - 383	1.32
CCAEANPPACYGTVLAEFQPLVEEPK	2779.28	4.05	384 - 409	4.28
TNCDLYEK	985.43	1.44	414 - 421	1.32
LGEYGFQNAILVR	1479.80	2.16	422 - 434	2.14
APQVSTPTLVEAAR	1439.79	2.10	439 - 452	2.30
LPCVEDYLSAILNR	1605.83	2.34	470 - 483	2.30
VCLLHEK	841.46	1.23	484 - 490	1.15
TPVSEHVTK	997.53	1.45	491 - 499	1.48
RPCFSALTVDETYVPK	1825.92	2.66	509 - 524	2.63
AETFTFHSDICTLPEK	1838.86	2.68	528 - 543	2.63
KQTALAELVK	1100.67	1.60	549 - 558	1.64
TVMDDFAQFLDTCCK	1736.73	2.53	570 - 584	2.47
AADKDTCFSTEGPNLVTR	1924.91	2.80	585 - 602	2.96
DTCFSTEGPNLVTR	1539.71	2.24	589 - 602	2.30
Totals:	39938.24	58.18	357	58.72

Figure 2-2. Identification of mouse serum albumin by in gel tryptic digest and mass spectrometry. Digested protein was analyzed on an LTQ-Orbitrap Mass Spectrometer and protein fragments were identified as MSA following a database search by BioWorks Software (Thermo Scientific).

A.

B.
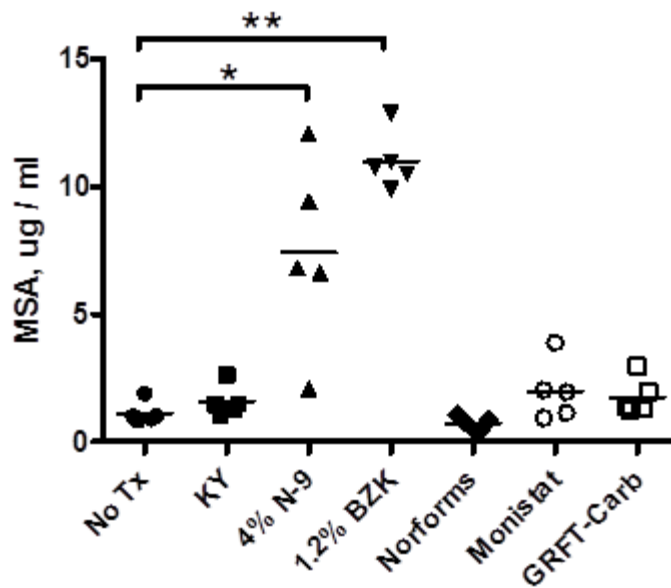
Figure 2-3. MSA concentrations in the CVL increase after exposure of mice to known epithelial disrupters. Mice were treated daily for 10 days with various OTC vaginal products or GRFT formulated in carbopol gel. MSA concentrations in the CVL were evaluated by immunoblot (A.) and ELISA (B.). Levels of MSA were significantly increased in mice treated with N-9 or BZK, compared with untreated mice. Bars indicate mean MSA concentration. Asterisks indicate $p < 0.05$ (*) or $p < 0.01$ (**), Dunn's Multiple Comparison Posttest.

A.   B.
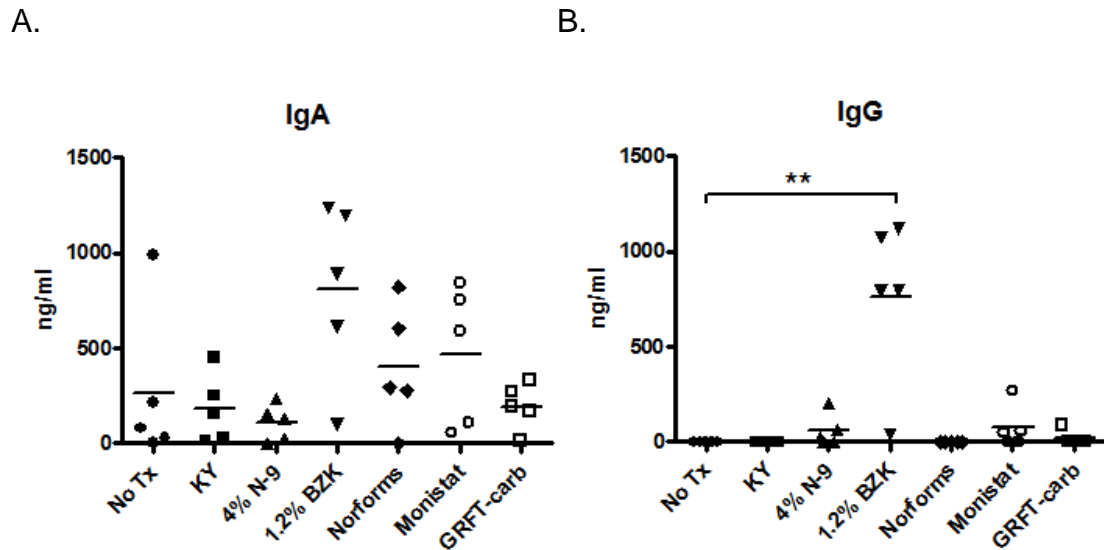
Figure 2-4. The effect of OTC vaginal products on immunoglobulin

concentrations in the CVL. Mice were treated daily for 10 days with various

OTC vaginal products or GRFT formulated in carbopol gel. Total IgA and IgG

concentrations in the CVL were evaluated by ELISA. Levels of IgA were not

significantly different from untreated animals in any of the groups. IgG levels

were significantly increased in mice treated with BZK, compared with untreated

mice. Bars indicate mean immunoglobulin concentration. Asterisks indicate p <

0.01, Dunn's Multiple Comparison Posttest.

A.

B.

IL1β

IL6
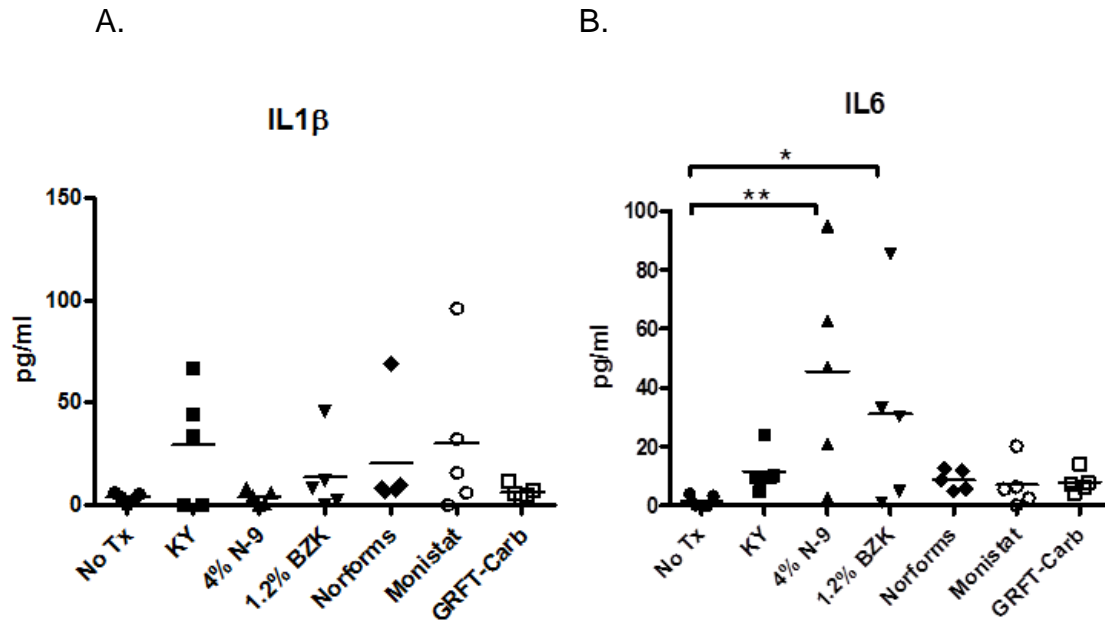
Figure 2-5. Concentrations of IL-6, but not IL-1β, are predictive of epithelial damage. Mice were treated daily for 10 days with various OTC vaginal products or GRFT formulated in carbopol gel. IL-1β and IL-6 concentrations in the CVL were evaluated by ELISA. Levels of IL-1β were not significantly different from untreated animals in any of the groups. IL-6 levels were significantly increased in mice treated with BZK and N-9, compared with untreated mice. Bars indicate mean cytokine concentration. Asterisks indicate $p < 0.05$ (*) or $p < 0.01$ (**), Dunn's Multiple Comparison Posttest.
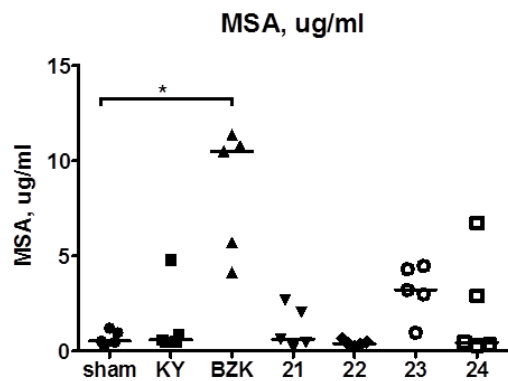
A.
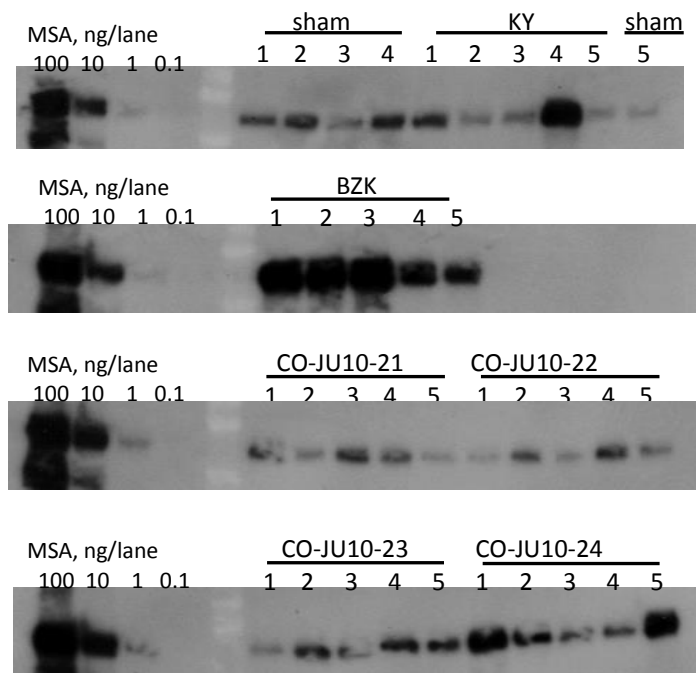
B.

Figure 2-6. **MSA levels in CVL are predictive of damage by BZK.** Western

blots (A.) and ELISA (B.) of cervicovaginal lavage samples from mice treated

with either KYJelly®, BZK, or a blinded panel of microbicides.

DISCUSSION

There is currently no effective vaccine against HIV, and thus other preventative measures must be used to thwart infection. Sexual transmission of the virus is the primary way in which it is spread, and microbicides which can be used prior to sexual intercourse may be an important tool for prevention, especially for women who cannot negotiate for condom use. The microbicides field has faced some setbacks, however, stemming from a number of products which failed to prevent the spread of HIV, or showed a trend towards harm, in several clinical trials. These failures were due at least in part to the lack of predictive preclinical safety data. In vitro assays are useful to a degree but lack the complexity of an intact reproductive system. A simple in vivo screening method for epithelial damage that could be employed in concert with other preclinical assays would be especially useful for the microbicides field.

The over-the-counter spermicide nonoxynol-9 (N-9) was tested extensively in clinical trials as a microbicidal candidate. Unfortunately it was not effective in preventing HIV-1 and other sexually transmitted infections at low frequency of use. With more frequent use (>3.5 applicators per day), a statistically significant increase in the rate of HIV-1 seroconversion was observed. Additionally, N-9 use resulted in the increased acquisition of multiple types of human papillomavirus (HPV) [47]. These resulting HIV and HPV infections were thought to be due to N-9 induced epithelial disruption. Mesquita et al provided further evidence for this by showing that both N-9 and cellulose

sulfate induce a reduction in the transepithelial electrical resistance and downregulation of tight junctional proteins [40].

MSA is the most abundant protein in serum. Its presence in vaginal washes at elevated concentrations may indicate damage to the vaginal epithelium. In the initial experiment, mouse serum albumin was identified by in-gel tryptic digest and mass spectrometry as the most prevalent protein in the cervico-vaginal lavage samples from mice treated with N-9. Faint bands at 68 kDa were observed in the mice treated with KY Jelly[®] as well, however they were not nearly as prominent as the bands in the N-9 treated group. It's likely that MSA leaked into the vaginal lumen because of the loss of tight junctions between the epithelial cells in the N-9 treated mice.

In the following experiment, mice were treated with a variety of over-the-counter vaginal products and formulated GRFT. The CVL samples were analyzed by western blot and ELISA, and the amount of MSA was compared with the untreated mice. As expected, the CVL of N-9 treated mice had a high concentration of MSA. The mice treated with Benzalkonium Chloride also had elevated levels of MSA in the CVL. This is unsurprising because BZK is a known epithelial disrupter [48,49]. Spermicides containing BZK are not sold in the United States due to the risks associated with its use, but are still available in other countries. The other products tested – KY Jelly[®], Norforms[®] deodorant suppositories, Monistat[®] anti-fungal cream, and formulated GRFT – had levels of MSA that were not significantly different than the untreated mice. The Norform-treated mice showed a trend toward lower levels of MSA than the untreated

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mice. This may be due to the consistency of the product. To make the Norform

product, which is supplied as a solid suppository, amenable to pipetting the

product was melted at $37^{o}C$ then mixed with 3% carboxymethylcellulose (CMC)

until it no longer solidified at room temperature. The resulting admixture was

thick and viscous, possibly producing a dense barrier along the vaginal

epithelium which resulted in reduced transudation of proteins across the vaginal

epithelium.

Levels of total IgA and IgG were evaluated in these mice as well, because

these immunoglobulins are also abundant in serum and an increased

concentration in the CVL may provide an additional marker for epithelial damage.

Both immunoglobulins were elevated in the CVL of mice treated with BZK, but

not for N-9. This indicates that presence of high concentrations of IgA and IgG

are predictive of gross damage to the epithelium, but fails to predict minor

irritants which still impact HIV acquisition.

Local production of the pro-inflammatory cytokines IL-1β and IL-6 have

been associated with decreased safety profile of microbicides [50,51]. In this

study, levels of IL-1β were similar among all treatment groups. IL-6 levels were

significantly different from untreated mice in the N-9 and BZK groups.

Surprisingly, the N-9 treated mice had higher levels of IL-6 in the CVL than the

more damaging BZK.

In the final experiment, mice were treated with KY Jelly, BZK, or one of

four blinded microbicidal products provided by CONRAD. Levels of MSA were

analyzed by ELISA and western blot, and these were compared to levels for

sham-treated mice. Again, the concentration of MSA in the CVL was highest for

BZK-treated mice. By ELISA, the group treated with CO-JU10-23 had the highest

overall levels of MSA of any of the groups treated with blinded microbicides, but it

was not significantly different from the sham-treated mice. The ELISA results

were similar to the data obtained from immunoblotting. BZK-treated mice

showed the highest levels of MSA by western blot, followed by mice treated with

either CO-JU10-23 or CO-JU10-24. These microbicides have not yet been un-

blinded to us by CONRAD, but it is likely that one of these gels may be Cellulose

Sulfate, which is known to perturb the vaginal epithelium.

One limitation with analyzing protein content and concentration in the CVL

is that there is no appropriate "loading control" to which data can be normalized.

Unlike analyzing cell lysates, in which data can be standardized to the number of

cells plated and the housekeeping protein concentrations expected with that

number of cells, the vaginal vault is an open system into which serum proteins

can enter. Because GRFT is a protein-based drug, and because many markers

for damage are proteins, it is inappropriate to normalize data to total protein

concentration. Thus all analyses must be done based on the assumption that the

volume of CVL obtained is the same for each mouse, and that the samples are

representative of the proteins present in the vaginal canal along the epithelial

surface. A solution to this problem remains an issue for the microbicides field.

In addition to identifying compounds which damage the epithelium of mice,

increased levels of serum albumin may be useful for predicting damage in

humans. The presence of certain sexually transmitted infections or

microtraumas which disrupt epithelial integrity are associated with an increased

risk of HIV acquisition. Measuring levels of serum albumin in women may

provide a facile method of identifying women who are at higher risk of infection,

especially in resource-limited settings where colposcopy is not always available.

Continued research into this area is needed.

CHAPTER 3

IMMUNOGENICITY OF VAGINALLY- AND SYSTEMICALLY- ADMINISTERED GRFT

Currently there is no vaccine to prevent the spread of HIV-1. In the absence of a vaccine, there has been increasing interest in topical microbicides which can impede the spread of HIV-1 and other sexually transmitted infections (STIs). Ideally a microbicide that is used for prevention of STIs should not activate the innate or adaptive immune response. However GRFT, like all protein-based drugs, may be immunogenic, and it is important to fully understand the consequences of any immune response in terms of safety and drug efficacy.

The female genital tract is an established site of induction of adaptive immune responses [52]. Systemic and local immune responses to Cholera Toxin B subunit (CTB) were evaluated in women following administration via the oral, rectal, or vaginal route [53]. Vaginal IgA and IgG was highest in vaginal secretions following vaginal administration, and all routes of administration resulted in higher concentrations of CTB-specific systemic IgA and IgG than in

pre-immune serum. However, it should be noted that there was great variation in the magnitude of antibody response in vaginal secretions among the women, possibly due to administration of the antigen at different phases of the menstrual cycle [54].

In laboratory rodents, immune responses vary throughout the estrous cycle. During diestrus, the immune response is dampened in the upper genital tract and is enhanced in the lower tract, presumably to facilitate fertilization and prevent infection in the pregnant doe [55,56]. Dendritic cells, MHC class II+ cells, and antigen-presenting cells are present in increased numbers and the vaginal epithelium is thinned during the progesterone-dominated diestrus phase, which may enhance an immune response to vaginally-applied products [55,56]. Injection with depot medroxyprogesterone (Depo-Provera) mimics this effect. The work presented in this chapter will detail the adaptive immune response to intravaginally applied GRFT in Depo-Provera treated mice.

MATERIALS AND METHODS

Animal Experiments

Six to eight week old BALB/c (Jackson Labs) or SKH1-E (Charles River) hairless mice were injected subcutaneously with 3 mg Depo-Provera (Pfizer) 3 or 4 days prior to each experiment. Animals were euthanized by CO_2 asphyxiation and death confirmed by cervical dislocation.

To evaluate the effect of route of administration on local and systemic antibody levels, animals were treated weekly with either GRFT or GRFT^{lec-}. 1.2 µg of each protein were diluted in 1X PBS and injected subcutaneously (s.c.), or 12 µg of GRFT or GRFT^{lec-} mixed in carbopol gel and administered intravaginally (ivag) using a positive-displacement pipette. A total of 6 treatments were administered. Samples were collected one week after the final treatment.

To compare the antibody response to two doses of two lectins, mice were administered six weekly intravaginal doses of either 1.2 or 12 µg of GRFT, GRFT^{lec-}, or cyanovirin-N (CV-N) mixed with KY Jelly.

Sample Collection

CVL: Cervico-vaginal lavage (CVL) samples were collected by dispensing and aspirating 150 µl of 1X PBS containing protease inhibitor 10 times, and then repeating for a total of 300 µl CVL. The lavage samples were centrifuged at 800 rcf for 10 minutes, and the supernatant was stored at -80°C.

Serum: Terminal bleeds were collected via cardiac puncture after mice were euthanized by CO2 asphyxiation and cervical dislocation. Blood collected in serum separator tubes clotted for 30 minutes at room temperature, then was centrifuged for 5 minutes at 6000 rcf. Serum was stored at -80°C.

Splenocytes: Spleens were removed and placed in RPMI 1640 containing 2% Fetal Bovine Serum (FBS). The tissue was crushed on metal mesh with forceps, and the cell suspension was centrifuged at 450 x g for 5 minutes. The cells were resuspended in ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) and incubated for several minutes. Additional medium was added to the suspension and centrifuged again. The resulting cell pellet was resuspended in RPMI 1640 containing 10% FBS and filtered through a 0.2 micron filter.

ELISA

Plates were coated with 0.25 µg/ml gp120. Wells were blocked with 5% (w/v) non-fat dry milk in 1X PBS-T. Following blocking, 2 µg/mL GRFT or CV-N in 1X PBS was added and incubated for 1 hour. Test serum or CVL was diluted in 1X PBS and incubated for 1 hour at RT. Pre-immune serum or CVL served as the background control. Goat anti-Mouse IgG-HRP or IgA-HRP was added. The plate was developed with KPL SureBlue TMB Microwell Peroxidase Substrate, and the reaction was stopped with addition of 1N H_2SO_4. The plate was read at an OD of 450nm. Antibody titers are represented as the reciprocal of the dilution that gave a reading of twice background.

ELISpot

IL-4 and INFγ capture antibodies were diluted in 1X PBS and coated on ELISpot plates (Millipore) overnight at 4^oC per the manufacturer's instructions (eBiosciences). Wells coated with PBS only were used as background controls. Plates were washed twice with 1X PBS and blocked for 1 hour at room temperature with RPMI 1640 containing 10% FBS. After washing the plates 3 times in 1X PBS, GRFT or CV-N was added at 10 μg/mL in RPMI 1640/10% FBS. Five-fold serial dilutions, starting at 107 cells per mL, were added in quadruplet, so that 3 wells at each dilution contained capture antibody and 1 well served as a background control. Cells and stimulants were co-incubated for 24 hours at 37^oC. Plates were washed and biotinylated detection antibody was added and incubated for 2 hours. Plates were washed extensively in PBS-T, then Avidin-HRP was added and incubated for 45 minutes. Following extensive washings with both PBS-T and PBS, the plates were developed with freshly prepared AEC substrate for 60 minutes. The reaction was stopped with water and the plates allowed to dry. Plates were imaged on an Immunospot (Cellular Technology, Ltd.) with Immuno Capture 6.3 software, and spots were counted using ImmunoSpot 5.0 Pro DC.

Positron Emission Tomography

2.5 nmol DOTA-GRFT was mixed with ^{64}Cu in ammonium acetate buffer (100 μL, pH 8), and was incubated at room temperature for 15 minutes. The

labeled mixture was passed through a NAP-5 column, and the labeled protein

was eluted with PBS. The labeling yield was in the range of 80-90%. The

radiochemical purity was greater than 99% based on analytical HPLC analysis.

The labeled protein was mixed with carbopol gel and administered to SKH1-E

mice with a positive displacement pipette. The mice were anesthetized with

isofluorane, and the animals were imaged with a MicroPET rodent R4 scanner

from Siemens. Images were acquired with ASIPro and Analyze imaging software.

Statistics

Graphpad Prism software version 5.04 was used for graphing results and

statistical analysis. Data were analyzed by the Mann-Whitney test. A p-value of

<0.05 was considered significant.

RESULTS

Weekly doses of GRFT or GRFT^{lec-} were administered either subcutaneously or intravaginally for 6 weeks. Systemic and local antibody responses were analyzed by gp120 capture ELISA. Intravaginal administration of GRFT elicited significantly higher serum antibody titers than subcutaneous administration ($p = 0.0350$) (**Figure 3-1**). For GRFT^{lec-}, the trend was the same as for GRFT, but the difference was not statistically significant ($p = 0.2059$).

GRFT-specific IgA levels in the CVL were evaluated for the same mice (**Figure 3-2**). Surprisingly, the mean concentration of GRFT-specific IgA was higher for the subcutaneously-treated mice than for those treated intravaginally, however this difference was not statistically significant ($p = 0.5476$). Mice treated with GRFT^{lec-} had lower overall IgA antibody levels in the CVL, and there was no significant difference between the routes of administration.

To monitor the distribution of GRFT in the genital tract following intravaginal administration, GRFT was labeled with Cu-64 and mice were subjected to positron emission tomography (PET). Scans were taken at 1, 2, 7 and 31 hours after administration of the gel (**Figure 3-3**). GRFT was present in the vagina at all time-points. Surprisingly, labeled GRFT had traversed the cervix and entered the uterus within an hour. By the last time-point, labeled GRFT was observed in the peritoneum.

To further evaluate mucosal administration of GRFT, female BALB/c mice were administered six weekly intravaginal doses of either 1.2 or 12 µg of GRFT, GRFT[lec-], or cyanovirin-N (CV-N), another lectin that is also being developed as a potential microbicide. Antibody titers were measured in serum and CVL in a gp120-binding ELISA (**Figure 3-4**). Serum antibody titers were relatively high for all three groups treated with the high dose (12 µg of protein), but only animals treated with the low dose of GRFT (1.2 µg) also had high serum antibody titers. The difference between low and high dose was only statistically significant for mice treated with GRFT[lec-] (p = 0.0254). Mucosal IgA levels were fairly low, however mice treated with 12 µg of GRFT had higher CVL IgA levels than other mice. The difference between low and high doses of GRFT was not statistically significant. Likewise, increasing the dose of the microbicide did not result in statistically higher levels of IgG in the CVL for any of the groups.

To measure T-cell responses to GRFT or CV-N, splenocytes from treated animals were evaluated for cytokine release by ELISpot after stimulation with GRFT or CV-N (**Figure 3-5**). Overall numbers for both IL-4 and INFγ were low. Both low and high dose treatments of CV-N resulted in 2- to 4-fold higher number of interferon gamma-secreting cells than seen in mice treated with GRFT or GRFT[lec-]. Numbers of IL-4-secreting splenocytes elicited by treatment with the microbicides were dose-dependent, with IL-4 secreting cells only evident in the animals treated with the higher dose.
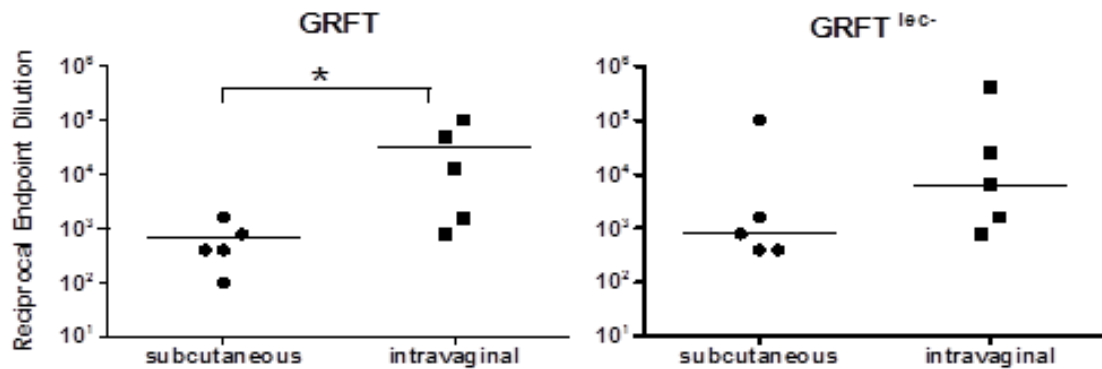
Figure 3-1. Intravaginal administration of GRFT, but not GRFT^{lec-}, induces higher titers of serum IgG antibodies than subcutaneous administration.
Mice were treated intravaginally or subcutaneously with either GRFT or GRFT^{lec-} weekly for 6 weeks. Anti-GRFT IgG titers in the serum were determined by gp120 capture ELISA. Bars indicate mean endpoint titers. Asterisk indicates p = 0.0350, Mann-Whitney test.
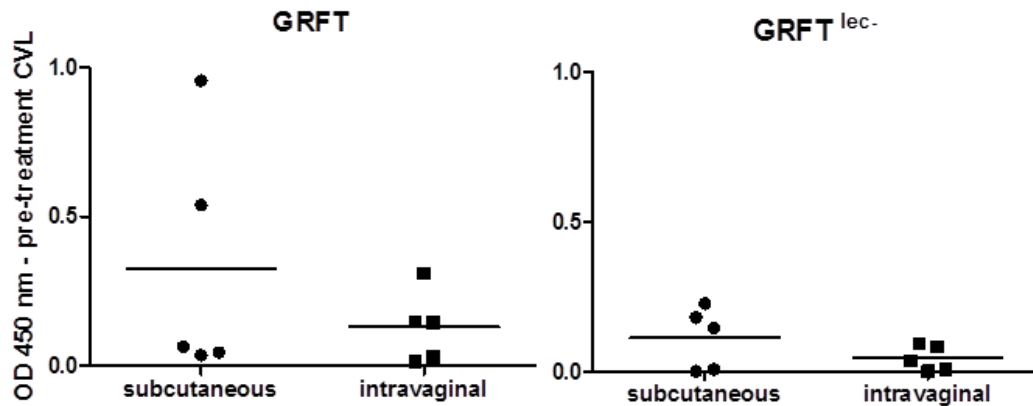
Figure 3-2. **GRFT-specific IgA concentrations in the cervico-vaginal lavages are low following either subcutaneous or intravaginal administration of GRFT or GRFT^lec-.**   Mice were treated intravaginally or subcutaneously with either GRFT or GRFT^lec- weekly for 6 weeks. Anti-GRFT IgA titers in the CVL were determined by gp120 capture ELISA. Bars indicate mean endpoint titers.
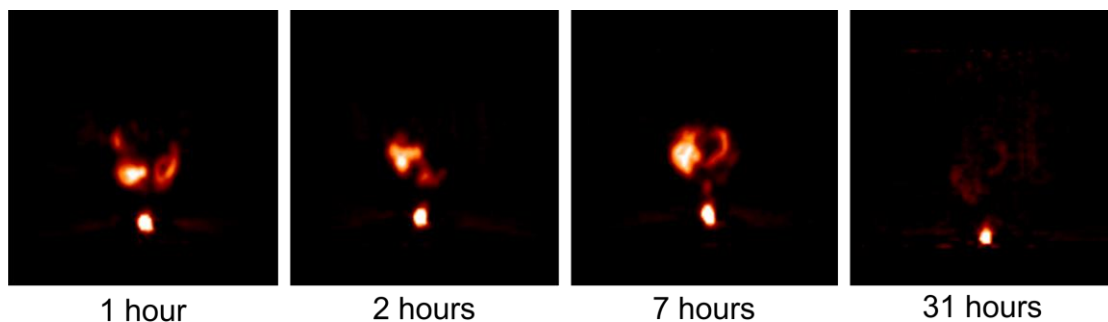
1 hour   2 hours   7 hours   31 hours

Figure 3-3. **Intravaginally administered Cu-64 labeled GRFT travels throughout the genital tract.** Cu-64 labeled protein was mixed with carbopol gel and administered to SKH1-E hairless mice intravaginally with a positive displacement pipette. Mice were imaged by positron emission tomography (PET) at 1, 2 7, and 31 hours after administration.

A.

Antigen-specific serum IgG

B.

Antigen-specific IgA in CVL

C.

Antigen-specific IgG in CVL
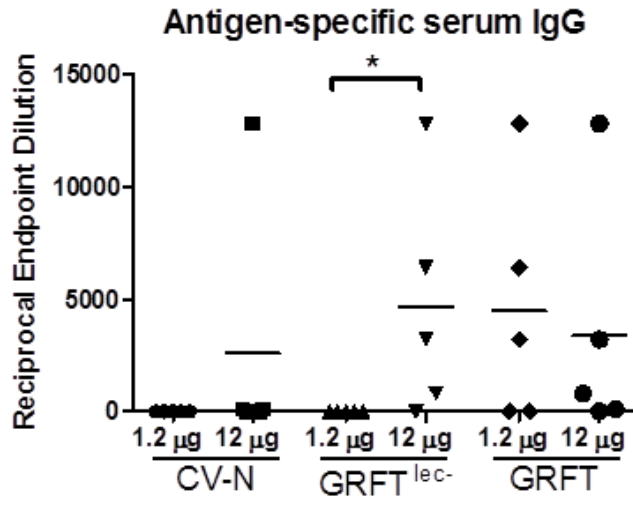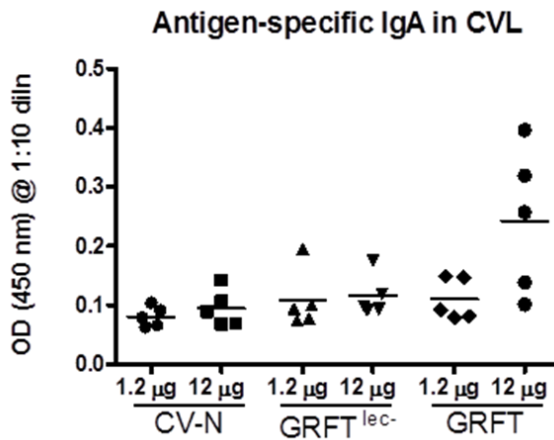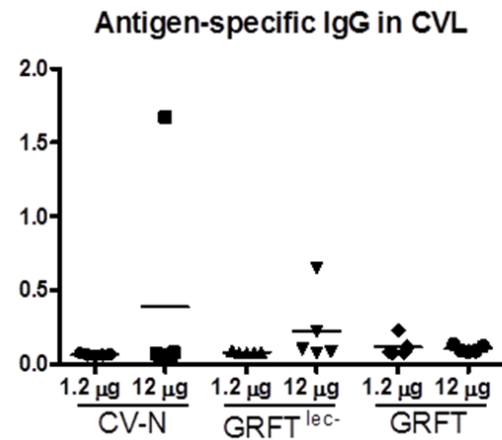
Figure 3-4. **Antigen-specific levels of serum IgG (A.), or mucosal IgA (B.)
and mucosal IgG (C.) in cervicovaginal lavages.** Female BALB/c mice were
administered six weekly intravaginal doses of either 1.2 or 12 µg of GRFT,
GRFT[lec-], or cyanovirin-N (CV-N). Antibody titers were measured in serum and
CVL in a gp120-binding ELISA.
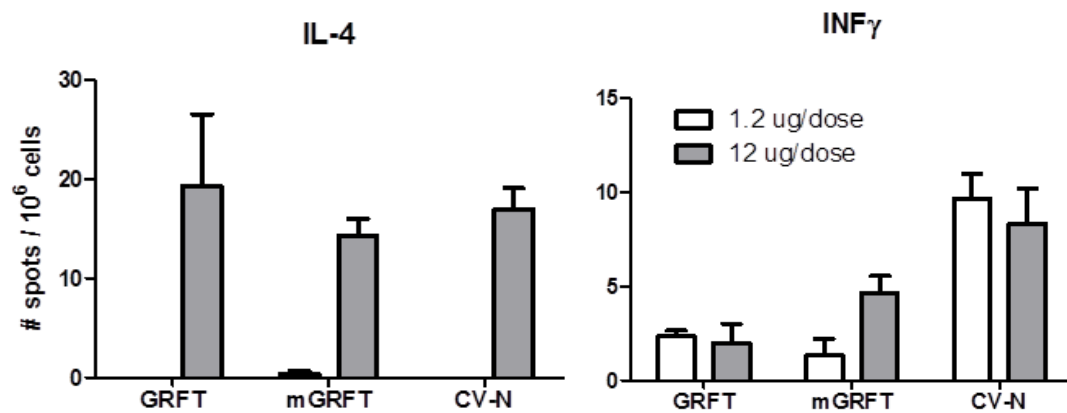
Figure 3-5. **T-cell responses to GRFT or CV-N following stimulation of splenocytes from treated animals.** To evaluate the T-cell responses, 1 week after the final dose splenocytes from the immunized mice were stimulated with antigen for 2 days, and the number of cells secreting IL-4 and INFγ were counted. Bars indicate mean + SEM.

DISCUSSION

GRFT is a promising candidate as a vaginal microbicide. To date, its safety profile indicates that it is not mitogenic, does not cause inflammatory cytokine release, and does not decrease cell viability [24,57]. However, like all lectins it has the capacity to be immunogenic. To understand GRFT's ability to raise an immune response, mice were exposed to GRFT without adjuvant, either via the subcutaneous or mucosal (intravaginal) route.

A subcutaneous dose of 1.2 ug of GRFT had been used previously [58] and because many mucosal antigens are taken up at very low levels [52], we selected a 10-fold higher dose for mucosal administration. Repeated intravaginal exposure of mice to either GRFT or GRFT[lec-] did not produce high titers of IgA or IgG antibodies to GRFT in the vaginal vault. In fact, two of the mice injected subcutaneously with GRFT had the highest titers of IgA antibodies at the mucosal surface. Surprisingly, intravaginal administration of GRFT resulted in higher titers of serum IgG antibodies than subcutaneous administration did. From data collected using positron emission tomography, we found that GRFT travels readily through the female reproductive tract and into the peritoneum. This may explain why intravaginal administration of GRFT results in high titers of systemic anti-GRFT antibodies.

To further evaluate mucosal administration of GRFT, female BALB/c mice were administered six weekly intravaginal doses of either 1.2 or 12 µg of GRFT, GRFT[lec-], or cyanovirin-N (CV-N). Serum antibody titers were relatively high for

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all three groups treated with the high dose (12 µg of protein), but only animals treated with the low dose of GRFT (1.2 µg) also had similarly high serum antibody titers. The reason for low doses of GRFT eliciting high systemic antibody titers is unclear. GRFT, but not GRFT[lec-], binds the vaginal epithelium [57]. This binding may enhance the retention time of lectin-based microbicides *in vivo*, thus increasing the amount of drug that reaches the systemic immune system.

Induction of anti-GRFT antibodies could impair its utility as a microbicide by interfering with its ability to bind to glycoproteins. This could occur by an antibody that binds directly to the carbohydrate-binding moieties on GRFT, or may be the result of steric hindrance. Further testing will need to be completed to evaluate what, if any, effect these specific antibodies have on GRFT's antiviral activity.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Our GRFT-based gel product presents an appealing innovation in the microbicide field. Because it is a small protein, and not orally bioavailable, it is unlikely that GRFT will be developed as part of the normal antiretroviral treatment regimen, making it available for prophylaxis without compromising population-level antiretroviral efficacy through promoting drug resistance. This is an important differentiating factor for GRFT and other protein- and peptide-based microbicides since there are serious concerns about whether antiretrovirals that are used in treatment regimens are appropriate for use in prophylaxis, since the risk of resistance due to suboptimal viral suppression compromises the therapeutic use of not only the specific drug, but also members of the same class of drug [59,60]. Recently, Schader et al. demonstrated that for the leading non-nucleoside reverse transcriptase inhibitor (NNRTI) microbicide candidate, Dapivirine (DAP), evolution of viral resistance associated with incomplete viral replication suppression resulted in cross-resistance to Nevirapine (NVP), a related NNRTI that is vitally important in HIV therapy and perinatal HIV transmission prophylaxis protocols in sub-Saharan Africa [61]. We have

demonstrated that GRFT exhibits significant antiviral synergism with leading

small molecule ARV Tenofovir (TFV), Maraviroc, Enfuvirtide [62], and Raltegravir

(presented at CROI, 2012).

Amongst all protein- and peptide-based microbicide candidates, GRFT

has many outstanding advantages. Foremost is its mid-picomolar potency

against a broad range of HIV-1 strains, its activity against HSV-2 [63,64], and

against Heptatitis C virus (HCV) [65]. Although HCV is not primarily a sexually

transmitted pathogen, there is increasing evidence that it may be sexually

transmitted in some HIV positive populations. GRFT has remarkable

environmental stability, with a thermal melting temperature in excess of 78°C,

and strong resistance to digestion by human and bacterial proteases [24,66,67].

Our GRFT carbopol gel formulation shows good API release kinetics and product

stability. In preclinical tests, the API and gel product have an excellent safety

profile [24,57].

Perhaps the most concerning theoretical safety issue for protein- and

peptide-based microbicides is the potential for immunotoxicity. Xenogenic

proteins, such as GRFT, may induce inflammatory immune responses.

Moreover, there is a risk that on repeated use, anti-drug immunoglobulins could

lead to neutralization of the lectin's activity. These risks have generally not been

addressed by other groups who propose lectins as microbicides. GRFT is not

mitogenic, does not activate T-cells, and induces very little alteration in secretion

of inflammatory cytokines in treated PBMC from multiple human donors [24,57].

Similarly, we have not observed production of inflammatory cytokines in mucosal

secretions of rabbits treated with unformulated API [24] and mice treated with GRFT gel formulations, but we have confirmed that GRFT and CV-N both induce systemic antibody responses and very low titers of binding mucosal antibodies after vaginal administration to Depo-Provera treated mice, which have substantially thinned vaginal mucosa after hormonal treatment.

It is important to note that all of the *in vitro* experiments in the previous chapters were performed in the context of Depo-Provera (medroxyprogesterone acetate) pre-treatment. This is done primarily to thin the vaginal epithelium and increase susceptibility to drug treatment. However, it also reflects the fact that Depo-Provera and other injectable progesterone drugs are a major form of contraceptives used by women worldwide, especially in developing countries [68,69]. The use of hormonal contraceptives by women at high risk of HIV acquisition is somewhat controversial. Hormonal contraception is vitally important for women to have control over their fertility. Some studies have associated the use of hormonal contraceptives with an increased risk in HIV transmission [70,71,72], while others have not [72,73]. This uncertainty prompted the World Health Organization to review the literature and consider whether the previous guideline *Medical eligibility criteria for contraceptive use, Fourth edition 2009* should be altered in light of these published studies [74]. Ultimately the review panel deemed injectable hormonal contraceptives safe, with a strong clarification as to the potential risks for women at high risk of HIV infection.

Future work for this project includes further evaluation of GRFT's acceptability as a protein based microbicide. We will assess its ability to alter the innate immune response within the vaginal mucosa by measuring the number of HIV target cells after single or repeated doses of formulated gel. We will determine the consequences of anti-GRFT antibodies following repeated mucosal administration, assaying reduction of HIV gp120 binding associated with GRFT antibodies and neutralization of GRFT's HIV pseudovirus neutralization activity. We will also evaluate the impact of an antibody response to GRFT on its ability to protect mice from HSV-2 challenge, as well as the impact of an immune response to GRFT on gene and protein expression of key factors involved in HSV-2 infection. These future studies will provide a comprehensive overview of the innate and adaptive immune responses to GRFT, as well as any resulting consequences of that response.

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EDUCATION

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Employment

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Senior Research Technologist
- Design and implement a neutralization assay for evaluation of HPV vaccines
- Design vaccine trials and write IACUC protocols
- In vivo toxicology research, including development of a novel assay for epithelial disruption at mucosal surfaces
- In vivo immunology research, including the design of small animal experiments and evaluation of antibody responses by ELISA
- Laboratory and financial management
- Train post-docs and graduate students

Large Scale Biology Corporation (formerly Biosource Technologies, Inc.) – Vacaville, CA, 8/99 to 7/06

Associate Scientist, 6/02 to 7/06
- Produced and qualified virus fusion vaccines for internal projects and external clients, resulting in the extension of research agreements
- Developed protocols for bacterial expression of proteins
- Extensive protein design and purification from plants
- Vaccine design and animal study implementation
- ELISA and T-cell analysis of animal responses
- Acted as sequencing group leader, supervising one employee

- Implemented new sequencing protocols to reduce costs by $250,000 per year

Assistant Scientist, 9/00 to 6/02
- Optimized conditions for sequencing on the MegaBACE DNA sequencer
- Performed sequencing reactions and data tracking for >100,000 samples
- Created and optimized a high-throughput cell culture assay for a growth factor project

Laboratory Assistant, 8/99 to 9/00
- Prepared buffers and growth media for all scientific staff
- Responsible for hazardous waste collection and disposal

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Abstracts / Presentations

Oral presentations:
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3rd place, Research!Louisville, University of Louisville, 10/11
2nd place, Research!Louisville, University of Louisville, 10/10
International Papillomavirus Meeting, May 2009, Malmo, Sweden

Selected Meetings / Courses

- International Microbicides Conference, April 2012, Sydney, Australia.
- International Papillomavirus Meeting, May 2009, Malmo, Sweden.
- Principles of STD/HIV Research, July 2007, University of Washington, Seattle, Washington.
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PUBLICATIONS

Yaddanapudi K, Putty K, Rendon BE, Lamont GJ, Faughn JD, Satoskar A, **Lasnik A**, Eaton JW, Mitchell RA. Control of tumor-associated macrophage alternative activation by macrophage migration inhibitory factor. J. Immunol. 2013 Mar 15;190(6):2984-93.

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