SEE COMMENTARY

Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component

Barry R. O'Keefe[a], Fakhrieh Vojdani[b], Viviana Buffa[c], Robin J. Shattock[c], David C. Montefiori[d], James Bakke[e], Jon Mirsalis[e], Anna-Lisa d'Andrea[e], Steven D. Hume[f], Barry Bratcher[f], Carrie J. Saucedo[a,g], James B. McMahon[a], Gregory P. Pogue[b], and Kenneth E. Palmer[b,h,1]

[a]Molecular Targets Development Program, National Cancer Institute at Frederick, Frederick, MD 21702; [b]Intrucept Biomedicine, Owensboro, KY 42301; [c]Saint George's Hospital Medical School, University of London, London SW17 0RE, United Kingdom; [d]Department of Surgery, Duke University School of Medicine, Durham, NC 27708; [e]SRI International, Menlo Park, CA 94025; [f]Kentucky Bioprocessing, Owensboro, KY 42301; [g]SAIC-Frederick, Frederick, MD 21702; and [h]Department of Pharmacology and Toxicology and James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY 40292

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To prevent sexually transmitted HIV, the most desirable active ingredients of microbicides are antiretrovirals (ARVs) that directly target viral entry and avert infection at mucosal surfaces. However, most promising ARV entry inhibitors are biologicals, which are costly to manufacture and deliver to resource-poor areas where effective microbicides are urgently needed. Here, we report a manufacturing breakthrough for griffithsin (GRFT), one of the most potent HIV entry inhibitors. This red algal protein was produced in multigram quantities after extraction from *Nicotiana benthamiana* plants transduced with a tobacco mosaic virus vector expressing GRFT. Plant-produced GRFT (GRFT-P) was shown as active against HIV at picomolar concentrations, directly virucidal via binding to HIV envelope glycoproteins, and capable of blocking cell-to-cell HIV transmission. GRFT-P has broad-spectrum activity against HIV clades A, B, and C, with utility as a microbicide component for HIV prevention in established epidemics in sub-Saharan Africa, South Asia, China, and the industrialized West. Cognizant of the imperative that microbicides not induce epithelial damage or inflammatory responses, we also show that GRFT-P is nonirritating and noninflammatory in human cervical explants and in vivo in the rabbit vaginal irritation model. Moreover, GRFT-P is potently active in preventing infection of cervical explants by HIV-1 and has no mitogenic activity on cultured human lymphocytes.

AIDS | lectin | plant | sexually transmitted | tobacco mosaic virus

Improved prevention strategies are needed against the HIV/AIDS epidemic, including microbicides to protect the vaginal and rectal mucosa from HIV transmission. Effective prophylaxis of HIV transmission via anogenital epithelia should ideally block interaction between HIV-1 and CD4 and CCR5, as well as C-type lectin receptors on epithelial Langerhans cells and dendritic cells (1, 2). The entry or fusion inhibitor class of antiretroviral (ARV) drugs targets the initial entry stage of HIV infection. When formulated in simple gels, these effectively protect macaques against vaginal and rectal transmission of lentiviruses (3–7). Entry inhibitors are not commonly used in ARV therapy in resource-poor areas of the world and therefore show potential for use in prevention and treatment of multidrug-resistant viral infections in developing countries.

Many of the entry inhibitors that have been tested in preclinical efficacy trials in macaques are proteins and peptides produced using recombinant DNA technology. The broadly neutralizing mAb IgG1b12 (3), fusion inhibitor C52 (4), RANTES analogue PSC-RANTES (8), and lectin cyanovirin-N (CV-N) (6) all protected animals against vaginal infection with highly pathogenic simian HIV (SHIV). As components of microbicides, these agents do not carry substantial risk of systemic side effects because they are unlikely to be absorbed efficiently. Despite the promise that biologic drugs have as HIV prophylactics, their practical application as topical microbicides is hampered by high production costs. It is unlikely that any manufacturing system reliant on growth in sterile conditions can be competitive with the price of a male condom, which is necessary if the product is to be available for use by those at risk for sexual transmission of HIV. Consequently, production at agricultural scale in field crops is considered the most promising means of manufacturing recombinant proteins for use as active ingredients in topical microbicides (9, 10).

Among the most potent HIV entry inhibitory molecules are lectins that target the high mannose N-linked glycans (NLG) displayed on the surface of HIV envelope glycoproteins. HIV uses glycans to shield important domains of the envelope protein from neutralizing Abs and to facilitate the infection of Langerhans cells and dendritic cells (2). Of the biologically based entry inhibitors, the red algal protein griffithsin (GRFT) has the most potent anti-HIV inhibitory activity, with an average EC_{50} of 40 pM (3–5, 11). GRFT targets the terminal mannose residues on high mannose oligosaccharides and has a total of 6 carbohydrate binding sites per homodimer (12, 13), which likely accounts for its unparalleled potency. Inactivation of HIV-1 by GRFT happens almost immediately on contact with the virus (14). The antiviral potency of GRFT, coupled with its lack of cellular toxicity, exceptional environmental stability (resistance to a broad range of pHs and temperatures nearing the boiling point), and stability in macaque cervicovaginal lavage fluid, makes GRFT an ideal active ingredient of a topical HIV microbicide (11, 14). In support of this concept, gel-formulated CV-N, another antiviral lectin, protected pigtailed macaques against high-dose intravaginal and intrarectal challenge with pathogenic SHIV 86.9P (6, 7).

We report that GRFT accumulates to a level of more than 1 g of recombinant protein per kilogram of *Nicotiana benthamiana* leaf material when expressed via an infectious tobacco mosaic

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Conflict of interest: F.V., G.P.P., and K.E.P. are members of Intrucept Biomedicine, which is commercializing plant-expressed GRFT. B.R.O. and J.B.M. are listed as inventors on patent applications related to GRFT.

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[1]To whom correspondence should be addressed. E-mail: kepalm02@gwise.louisville.edu.

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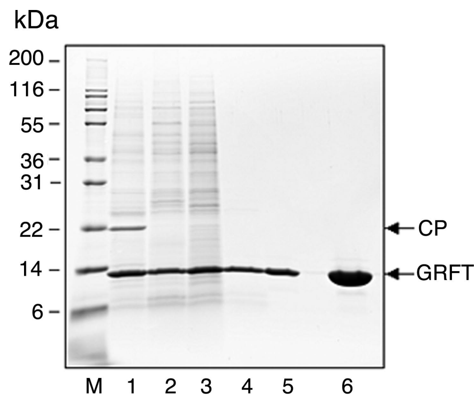

Fig. 1. A summary of the purification of GRFT-P from *N. benthamiana* plants infected with an rTMV vector expressing GRFT cDNA. The SDS-PAGE gel shows molecular weight marker in lane M. The initial GJ extract is shown in lane 1; the ceramic membrane permeate is shown in lane 2; lane 3 shows the UF concentrate; lane 4 is the SP-Sepharose column eluate; lane 5 contains the reversed-phase column chromatography eluate; and lane 6 shows 10 μg of the final GRFT-P product.

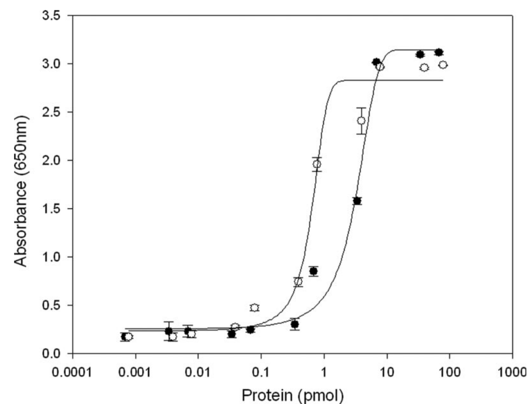

Fig. 2. Comparison of HIV-1_{IIIB} gp120 binding GRFT-E and GRFT-P. HIV-1_{IIIB} gp120 was bound to the wells of a 96-well plate and subsequently incubated with various dilutions of either GRFT-E (filled circles) or GRFT-P (open circles). Binding was visualized by HRP-labeled anti-GRFT rabbit polyclonal Abs and measured by absorbance at 650 nm. All test samples were measured in triplicate.

virus (TMV)-based vector (15), one already in use manufacturing proteins evaluated in human clinical trials (16). We produced more than 60 g of pure rGRFT in *N. benthamiana* grown in a single 5,000-square-foot enclosed greenhouse. In further validation of the feasibility of using plant-produced GRFT (GRFT-P) as the active pharmaceutical ingredient of a vaginal microbicide, we demonstrate that this product has broad and potent activity against a panel of primary sexually transmitted HIV-1 isolates from clades A and C, prevalent in sub-Saharan Africa and the Indian subcontinent, as well as against HIV-1 clade B viruses prevalent in the developed West. We also show that GRFT-P inhibits infection of human cervical explant tissues by HIV-1. It does not induce production of any of the proinflammatory cytokines known to recruit HIV-1 target cells to the vaginal mucosa and promote HIV-1 replication in infected cells. Furthermore, GRFT-P was also tested in the rabbit vaginal irritancy (RVI) model for topical microbicides and showed an acceptable safety profile at concentrations >10,000-fold higher than its antiviral EC_{50}.

Results

Production of rGRFT in Plants. We inserted a synthetic cDNA (GenBank no. FJ594069) encoding the 121 aa of GRFT (11) into a TMV vector in which GRFT is expressed under the control of a duplicated coat protein subgenomic promoter (15). *N. benthamiana* seedlings were inoculated with infectious in vitro RNA transcripts and rTMV inoculum purified from the virus-infected plants. This inoculum was used to infect more than 9,300 *N. benthamiana* plants in a greenhouse. The infected leaf biomass was processed 12 days after inoculation to extract GRFT-P. GRFT-P is the most abundant protein in a pH 5.5 extract from the plant material (Fig. 1, lane 1); the major contaminant is TMV coat protein.

The coat protein was removed by filtration through a ceramic membrane (Fig. 1, lane 2), and GRFT-P was purified by ion exchange chromatography over SP-Sepharose resin using ultrafiltration-diafiltration (UF-DF) concentrate (Fig. 1, lane 3). In total, 226.5 kg of infected plant material was processed. The first-column chromatography generated product that was >95% pure (Fig. 1, lane 4); a second reversed-phase purification generated product that was >99.8% pure (Fig. 1, lane 5 and overloaded in lane 6), with a final recovery of 30% of the *in planta* level of 1 g/kg. An additional 15–20% of the product could be recovered by reprocessing the initial fibrous material remaining after the first extraction (data not shown).

The average molecular mass of the purified protein was determined by MALDI-TOF MS. The mass of the product matches exactly the predicted mass of GRFT with N-terminal methionine deleted and terminal serine acetylated (Fig. S1). Size exclusion chromatography followed by light scattering analyses (data not shown), as well as x-ray crystallography, confirmed that GRFT-P is a homodimer (12). Further analyses of the purified product determined that the final preparation of GRFT-P has a concentration of 21.9 mg/mL, was sterile, contained no residual TMV, and had extremely low endotoxin contamination of 0.0896 EU/mL, much lower than tolerated by the U.S. Food and Drug Administration for an injectable drug.

HIV Envelope Glycoprotein Binding by GRFT-P. The binding of GRFT-P to HIV-1 gp120 was determined in comparison with *Escherichia coli*–expressed GRFT (GRFT-E) by a plate-based binding assay using immunologic detection of GRFT bound to plate-immobilized gp120, as previously described (8). As shown in Fig. 2, GRFT-P bound to gp120 in a manner similar to GRFT-E, thereby indicating that the GRFT-P possessed essentially the same oligosaccharide-dependent binding properties as standard GRFT-E.

In Vitro Anti-HIV Activity of GRFT-E. Both native GRFT, isolated from the red alga *Griffithsia* sp., and GRFT-P were tested for their ability to inhibit the cytopathic effects of HIV-1_{RF} on T-lymphoblastic CEM-SS cells. Both proteins showed remarkably potent activity against HIV-1, with EC_{50} values of 0.054 nM for native GRFT and 0.156 nM for GRFT-P. The differences in EC_{50} values between GRFT and GRFT-P may be accounted for by differences in methods used to quantify the 2 molecules, or could be due to the fact that GRFT-P contains the non-native amino acid alanine at position 31, in place of an unknown amino acid in the native algal protein. We performed virus entry inhibition assays with Env-pseudotyped HIV-1 bearing envelopes derived from primary isolates from each of clades A, B, and C. The results of these assays are shown in Table 1. The GRFT-P-sensitivity phenotypes of the assay viruses may be compared with sensitivity to soluble (s)CD4 and the broadly neutralizing mAbs IgG1b12, 2G12, 2F5, and 4E10 listed in Table S1, containing data extracted from references 17–19. Notably, all isolates from clades A and C were resistant to neutralization by mAb 2G12, which recognizes a glycan epitope on gp120, but all

Table 1. Antiviral activity of GRFT-P against pseudoviruses bearing envelopes from primary sexually transmitted isolates of HIV-1

Virus name	Clade	Antiviral activity of GRFT-P in TZM-bl cells*	
		IC_{50} (μg/mL)	IC_{90} (μg/mL)
6535.3	B	<0.003	0.02
QH0692.42	B	<0.003	0.12
SC422661.8	B	<0.003	0.05
PVO.4	B	<0.003	0.05
Du156.12	C	<0.003	0.50
Du172.17	C	<0.003	0.08
CAP45.2.00.G3	C	0.01	0.57
CAP210.2.00.E8	C	<0.003	0.12
Q23.17	A	0.07	2.03
Q168.a2	A	0.12	3.45
Q461.e2	A	<0.003	<0.003
Q259.d2.17	A	0.15	6.95
SVA-MLV	Amphocontrol	>100.00	>100.00

*Values are the concentration at which RLU were reduced by either 50% or 90% compared with virus control wells (no test sample).

strains were nonetheless sensitive to inhibition by GRFT-P. The data in Table 1 show that for 8 of the 12 HIV-1 isolates tested, the IC_{50} for GRFT-P was <3 ng/mL, or 235 pM, the lowest concentration tested, and the IC_{90} was <0.6 μg/mL for all clade B and C isolates. One subtype C isolate (CAP45.2.00.G3) displayed an IC_{50} of 10 ng/mL or 735 pM. Subtype A isolates were relatively less sensitive to GRFT-P, although 1 isolate showed exquisite sensitivity, with an IC_{90} of <3 ng/mL. Of the subtype A isolates, there was a direct correlation between the degree of sensitivity to GRFT-P and the number of predicted NLG sites present in the external domain of the Env protein. The most sensitive strain (Q461; GenBank no. AF4077151) has 32 potential NLG sites, whereas the most resistant strain (Q259; GenBank no. AF407152) has only 23 potential NLG sites. Collectively, these data show that of all of the biologicals listed in Table S1, GRFT-P was the only molecule that inhibited every strain and that, in general, it is substantially more potent than any of the mAbs and sCD4.

Evaluation of GRFT-P in Human Cervical Explants. We evaluated the toxicity and efficacy of GRFT-P using well-characterized human cervical explant assays (20). In Fig. 3 we show that GRFT-P induced no significant perturbations in levels of an extensive panel of cytokines and chemokines in cervical explants treated with 2.0 μM GRFT-P (at least 10,000 times the average antiviral EC_{50}) for 2 h and then cultured overnight. Control tissue explants were treated with vehicle alone (PBS). Similar results were seen specifically for IL-8 after intravaginal treatment of rabbits with GRFT-P (Fig. S2).

Assay for Mitogenic Activity of GRFT-P. Because several lectins have been reported to induce activation and proliferation of lymphocytes at high concentrations (21), it was important to evaluate whether GRFT-P treatment induced proliferation of human lymphocytes. Human peripheral blood mononuclear cells (PBMCs) were exposed to GRFT-P for either 2 h or 3 days. Proliferation was assayed after 3 days in culture by measurements of ^{3}H incorporation. The data presented in Fig. 4 show that GRFT-P has no detectable mitogenic activity on human PBMCs.

GRFT-P Prevention of HIV-1 Infection in Human Cervical Explants. Human cervical explants were incubated with various concentrations of GRFT-P and infected with CCR5-tropic HIV-1 subtype B strain BaL according to previously published methods (20, 22, 23). We also assayed infection of cells that migrated from the explant into media to predict the effect the drug treatment would have on cell-to-cell transmission, such as dendritic cell–mediated infection of T cells. Fig. 5 shows the results of these experiments. GRFT-P was a highly potent infection inhibitor for cervical explants (Fig. 5*A*) and was at least 10-fold more effective at preventing infection than CV-N (24). With infection of migratory cells, GRFT-P showed impressive efficacy, with virtually no infection down to concentrations as low as 100 pM (Fig. 5*B*).

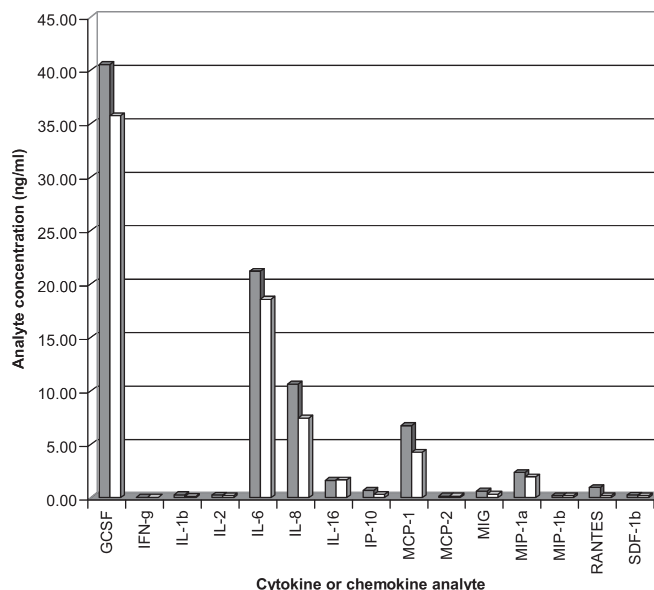

Fig. 3. Production of proinflammatory cytokines and chemokines in human cervical explants exposed to GRFT-P. Cervical explants were treated for 3 h with media alone (control, gray bar) and 2 μM GRFT-P (white bar). Expression of a panel of cytokines and chemokines was measured by Luminex-based immunoassays. GCSF, granulocyte colony-stimulating factor; IP10, Interferon inducible protein-10; MIG, monocyte induced by gamma interferon; SDF, stromal cell-derived factor.

RVI Assays with GRFT-P. The study was designed to have 5 animals per experimental group. The test article, GRFT-P, was formulated in PBS (pH 7.4). The treatment groups were (*i*) sham treated, (*ii*) vehicle only (PBS), (*iii*) 0.01% GRFT-P in PBS; (*iv*) 0.05% GRFT-P in PBS, (*v*) 0.1% GRFT-P in PBS, (*vi*) positive control nonoxynol-9 (N-9) contraceptive gel (Conceptrol, Ortho

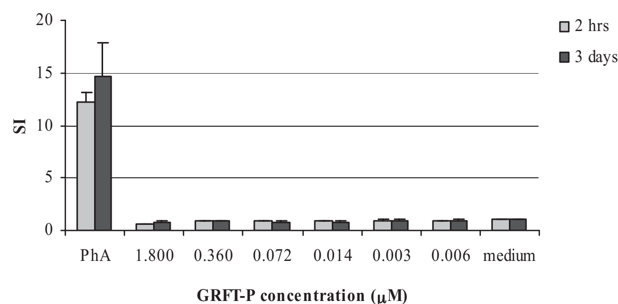

Fig. 4. Proliferative effects of GRFT-P on PBMCs. Proliferation was assayed in PBMCs after 3 days of culture. Cells were exposed to phytohemagglutinin A (PhA) or GRFT-P at different concentration for 2 h or 3 days. The stimulation index was calculated by dividing the mean cpm value of stimulated samples by the mean cpm of unstimulated ones.

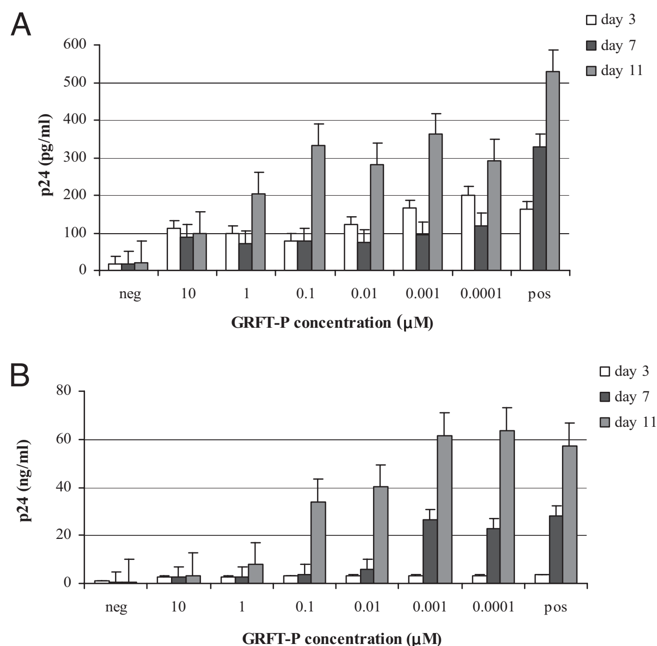

Fig. 5. Inhibition of HIV-1 BaL infection of cervical explants and dissemination by migratory cells. Infection of cervical explants was determined at postchallenge days 3, 7, and 11 by detection of p24 antigen in the tissue culture supernatants (*A*). GRFT-P was administered on day 0 and removed by washing after 2 h. Migratory cells were cocultured with PM1 cells, and infection was monitored by p24 ELISA at the same time points (*B*). GRFT-P was tested in cervical tissue deriving from 3 donors. Each condition was tested in triplicate. Data represent the mean ± SEM.

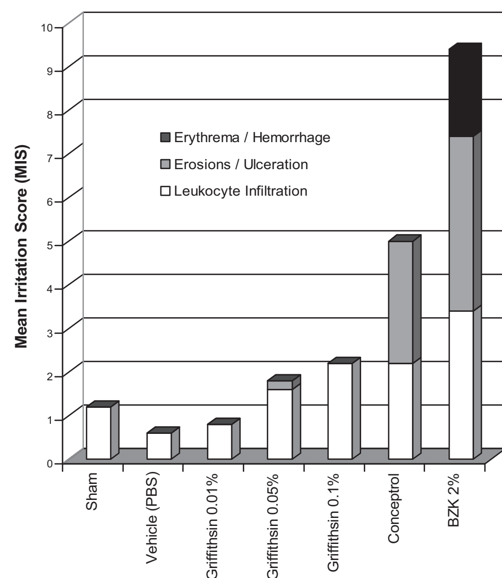

Fig. 6. RVI trial of GRFT-P. Bars represent the MIS derived from the sum of the contributions of the individual classes of pathology.

Options), and (*vii*) an additional positive control, benzalkonium chloride (BZK, 2%), which is known to be more toxic than N-9 in the RVI and also to induce production of proinflammatory cytokines in the rabbit vaginal epithelial tissues. A mean irritation score (MIS) of ≤8 is considered acceptable for clinical testing of vaginal products (25). The results of the RVI showed good safety profiles for all 3 concentrations of GRFT-P tested. No gross anatomic pathology associated with GRFT-P treatment was observed at any dose. The histopathologic MIS are illustrated in Fig. 6. The bars indicate the relative contribution of each different pathology assessment on the composite MIS. The MIS of 0.8 observed for 0.01% GRFT-P was lower than that seen in the sham-treated group (MIS = 1.2) and barely above that in the vehicle-only (PBS) group (MIS = 0.6). The leukocyte infiltrate observed in 0.1% GRFT-P–treated animals was the same as in animals treated with Conceptrol, which is not known to be immunogenic. Unlike Conceptrol, however, GRFT-P induced no epithelial ulcerations or erosions that account for the higher MIS (5.0) observed in the Conceptrol group. To further evaluate the inflammatory potential of GRFT-P on vaginal mucosa, vaginal swabs were taken from each test animal after 10 consecutive days of treatment, and the presence of IL-8 was measured by ELISA and quantified. Only the BZK treatment induced statistically significant increases in levels of proinflammatory cytokine IL-8; the Conceptrol, 0.01%, 0.05%, and 0.1% GRFT-P concentrations, sham, and PBS groups showed no significant increase in IL-8 levels (Fig. S2).

Discussion

Requirements for anti-HIV topical microbicide candidates include potency; broad-spectrum activity against the most prominent circulating strains of HIV; selectivity for viral or specific host cell targets; prevention of cell-to-cell transmission of HIV; stability both in transit and in vivo; bioavailability in target mucosa; no toxicity to mucosal surfaces, including direct irritation, immunogenicity, and mitogenicity; and finally, the ability to be produced in large quantities at minimal expense (26, 27). Several candidate microbicides have been put forward that meet only some of these criteria. Here, we describe the cost-effective, large-scale agricultural production of the antiviral protein GRFT-P, which we have now shown meets all these criteria.

The large-scale production of GRFT-P in *N. bethamiana* was achieved with a TMV-based expression system previously used for the expression of 16 individualized products evaluated in a phase I clinical trial and shown to be safe (16). What was remarkable with the GRFT-P results was the very high expression level of more than 1 g/kg leaf tissue, significantly higher than previously reported for GRFT-E in *E. coli* (28) and other biologically based HIV microbicide candidates mAb 2G12 (10) and CV-N (9) produced in plant systems. In addition, because of the biophysical characteristics of GRFT-P and the nature of the plant host, a very simple 3-step purification procedure was sufficient to produce protein of >99% purity with a yield of 300 mg/kg fresh weight. As yet, no protein- or peptide-based HIV entry inhibitors have been tested as microbicides in clinical trials, despite demonstrated efficacy in primate testing (3–7). Factors that may have limited their clinical development include manufacturing cost and availability of a suitable formulation. The facile purification and high expression levels of GRFT-P in *N. benthamiana* make this production system vastly more economically viable for the large-scale production of GRFT than fermentor-based production streams. This is a significant advantage for the future clinical development of this protein.

GRFT was originally isolated from the red alga *Griffithsia* sp. Native GRFT was shown to be potently active against HIV, with antiviral EC_{50} values in the picomolar range (8). Therefore, it was important to compare the biological activity of GRFT-P with that displayed by the native material and with that produced by *E. coli* (28). The ability of GRFT-P to inhibit HIV viral entry has been associated with its ability to bind directly to target oligosaccharides on viral envelope glycoproteins (8), so we first compared the ability of GRFT-P and GRFT-E for their ability to bind to the HIV-1 envelope gp120. As shown in Fig. 2, both proteins bound to HIV-1_{IIIB} gp120 readily, with GRFT-P actually showing slightly more affinity for the gp120 than the *E. coli*–expressed material. To more directly compare GRFT-P

with native GRFT, we tested both side-by-side in a whole-cell anti-HIV assay to determine their anti-HIV activity. As expected, both GRFT-P and native GRFT displayed picomolar anti-HIV activity well within the range previously reported for native GRFT (8). These data are similar to that previously reported for the relative activity of native, GRFT-E, and GRFT-P against SIV and SHIV strains (14).

Strains of HIV are grouped into distinct clades of HIV. Clade C and A strains predominate in sub-Saharan Africa and the Indian subcontinent, where the epidemic disproportionately affects women and where a female-controlled microbicide is most urgently needed. We tested GRFT-P against pseudoviruses derived from primary sexually transmitted isolates of HIV representative of clades A, B, and C and compared the efficacy of GRFT-P with that of other proteinaceous anti-HIV agents, including sCD4 and several mAbs. GRFT-P was active against all of the viruses tested, displaying greater potency and a much broader spectrum of activity than any other agents, including the carbohydrate-specific Ab 2G12, which binds to similar oligosaccharide epitopes (29). GRFT-P showed variability in its activity against clade A viruses (with EC_{50}s ranging from <235 pM to ≈10 nM), which could be correlated with the degree of glycosylation on these viruses.

GRFT-P was shown to prevent cell-to-cell transmission of HIV (8) and was also reported to be stable for at least 24 h in cervicovaginal lavages from macaques (14). GRFT-P is stable to a variety of physical conditions, including low pH, organic solvents, multiple freeze–thaw cycles, lyophilization, and reducing agents. GRFT resistance to the deleterious effect of these treatments could be related to its unusually high physical stability, reflected by its melting temperature of ≈80 °C (S. Shenoy-Kurian, unpublished results). Because the stability of clinically useful proteins is always a concern for in vivo efficacy, GRFT's demonstrated maintenance of anti-HIV activity after these physical insults is significant. In addition, GRFT's high melting temperature may provide many practical advantages for shipping and storage of this protein in resource-poor areas.

The most pressing concerns for potential anti-HIV microbicides are in vivo safety and efficacy. After the high-profile failures of first-generation microbicide products, we are cognizant that the highest priority for new microbicide candidates is to demonstrate ideal preclinical safety profiles. Many plant lectins stimulate lymphocyte proliferation in vitro and in vivo (21). Unlike many other products proposed as microbicides, GRFT-P possesses no measurable lymphoproliferative activity in vitro (Fig. 4). Before clinical testing can proceed, it is necessary to show that GRFT-P does not induce inflammatory responses in vaginal epithelial tissues. The standard test for safety of vaginal topical products required by the U.S. Food and Drug Administration is the RVI assay (25). Therefore, we evaluated GRFT-P in the RVI assay, finding that GRFT-P has an acceptable safety profile to proceed to clinical testing (Fig. 6). Furthermore, GRFT-P was shown to be safe in ex vivo human cervical explants (Fig. 3 and Table S1) and to effectively inhibit HIV-1 BaL infection of cervical explants and dissemination by migratory cells (Fig. 5).

To be useful as a microbicide, an ARV must meet stringent safety, efficacy, and affordability metrics. Recombinant protein–based ARVs have the potential to play a significant role in preventing transmission of HIV-1 but have to meet the challenge of low cost, large-capacity manufacturing, and environmental stability. GRFT-P meets all of these requirements and is now a validated candidate protein microbicide to bring toward clinical testing in humans.

Materials and Methods

Expression Vector Construction and Plant Infection. A synthetic cDNA (GenBank no. FJ594069) encoding the GRFT amino acid sequence was cloned into DN15, a TMV-based expression vector derived from the TMV-30b vector to create pITI003 (15). Wild-type *N. benthamiana* seedlings were grown with supplemental lighting to provide a 20-h light/4-h dark cycle in a greenhouse. Infectious transcripts of pITI003 vector RNA were generated by T7 polymerase-mediated in vitro transcription. Transcripts were inoculated on *N. benthamiana* seedlings (24 days after sowing) by gentle abrasion of the 2 uppermost fully expanded leaves. For virus passage, rTMV was isolated from plants infected with in vitro transcripts 7 days after inoculation.

Purification and Characterization of GRFT-P. Twelve days after inoculation, infected leaf and stem material was harvested. Plant biomass was passed through a disintegrator, and extraction buffer [30 mM acetate buffer (pH 4.0), containing 313 mM NaCl, 23 mM ascorbic acid, and 8 mM sodium metabisulfite] was added at a buffer/biomass ratio of 0.5:1 vol/wt. The biomass was then processed through a screw press, and aqueous homogenate ''green juice'' (GJ) was collected. The pH of the GJ was adjusted to 5.5. After equilibration GJ was pumped into a feed tank, and ceramic filtration was performed. The ceramic permeate was collected, and 2 additional ceramic filtration washes were performed to maximize protein recovery. The ceramic permeates were combined after sampling and adjusted to pH 7.0, then pumped into a UF feed/concentrate tank through a 5-μm filter. The UF feed was concentrated to 15.4 L, followed by DF using 20 mM phosphate buffer, pH 7.0 (DF buffer). DF buffer was added to the system to reduce the conductivity of the UF concentrate to 2.6 mS/cm. The UF-DF concentrate was filter sterilized using a 0.2-μm filter and stored at 4 °C. Before column chromatography, the pH of the UF-DF concentrate was adjusted to 4.0. UF-DF concentrate containing GRFT was loaded on a SP Sepharose Fast Flow (GE Healthcare) column equilibrated with 20 mM acetate buffer, pH 4.0 (solution A). After washing of the unbound proteins from the column, the GRFT-P was eluted with 100 mM NaCl and a linear gradient of 100–250 mM NaCl in buffer A. The pH of eluted fractions was subsequently adjusted to 7.0. Fractions were analyzed by SDS-PAGE and by MALDI-TOF MS. Protein was then run on a Source 15RPC column (GE Healthcare) equilibrated with 20 mM phosphate, pH 7.4 (solution B), and eluted using a 1.13% n-propanol gradient, followed by a 5.25% linear n-propanol gradient in solution B. Fractions containing >99.5% pure GRFT-P were pooled and diafiltered against PBS, pH 7.4. Purity and approximate molecular mass of GRFT-P was determined using SDS-PAGE. Coomassie blue–stained protein bands were quantitated using a densitometer. For accurate mass analysis of the GRFT-P molecule, we used MALDI-TOF MS. GP binding assays and in vitro anti-HIV assays with HIV-1 RF were performed as described previously (11, 28).

HIV Pseudovirus-Based Neutralization Assay. The antiviral activity of GRFT-P was measured as a function of a reduction in luciferase reporter gene expression after a single round of infection with Env-pseudotyped viruses in TZM-bl cells, as previously described (17, 19). TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Antiviral activity is reported as the sample concentration at which relative luminescence units (RLU) were reduced by either 50% or 90% compared with virus control wells after subtraction of background RLUs. Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by transfection of 293T cells and were titrated in TZM-bl cells as previously described (17). Clade A, B and C reference Env clones have been described previously (18, 19, 30). The broadly cross-neutralizing mAbs b12, 2G12, 2F5, and 4E10 were used as positive controls.

Proliferation Assay. PBMCs were isolated from blood by Ficoll-Paque separation. Cells were seeded at a concentration of 2×10^5 cells per well in triplicate and incubated with GRFT-P at different concentrations for either 2 h or 3 days. Positive control cells were incubated with 2 μg/mL phytohemagglutinin A. After 3 days, the cultures were pulsed for 16–18 h with 1 μCi per well of [H^3]-thymidine, and then intact DNA was harvested by membrane filtration. Incorporated radioactivity was measured by a scintillation counter (Beckman Coulter). The stimulation index was calculated by dividing the mean cpm value of stimulated samples by the mean cpm of unstimulated ones.

Culture and Infection of Human Genital Tissue Explants. Cervical explants culture was performed as previously described (31). Tissue was obtained, after consent, from patients undergoing planned therapeutic hysterectomy. Cervical tissue, including both epithelium and stroma, was cut into 3-mm^3 explants and cultured in RPMI medium 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Explants were pretreated for 2 h with GRFT-P at different concentrations before exposure to HIV-1_{BaL} (5×10^4 median tissue culture infectious dose) (for 2 h at 37 °C). Subsequently, explants were washed 4 times with PBS and cultured overnight in 96-well microplates. The next day, explants were transferred to

a new microplate and cultured for 11 days, with supernatant harvests every 3 days. Migratory cells, present in the overnight culture plate, were washed with PBS and cocultured with 4×10^4 PM-1 cells to assess virus transfer. Supernatants were harvested every 3 days. HIV-1 infection was determined by p24 ELISA in both culture supernatants.

Cytokine Detection. Tissue explants were exposed to different concentrations of GRFT-P for 2 h, then washed and cultured overnight with medium only. Supernatants were collected after overnight incubation. PBMCs were exposed to GRFT-P for either 2 h or 3 days, and cytokine release was assayed after 3 days of culture, in parallel with the proliferation assay. Culture supernatants were simultaneously assessed for the presence of the following cytokines: granulocyte colony-stimulating factor, granulocyte–macrophage colony-stimulating factor, IFN-γ, IFN-β, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IL-15, IL-16, Interferon inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-2, monocyte induced by gamma-Interferon (MIG), macrophage inflammatory protein-1alpha (MIP-1α), MIP-1β, RANTES, stromal cell-derived factor 1α, transforming growth factor β, and tumor necrosis factor α in a bead-based fluorometric assay according to the manufacturer's protocols (R&D Systems). Plates were read using the Luminex 100 system, and data were analyzed by Bioplex Manager 4.0 software (BioRad).

RVI Assay. Safety assessments of vaginal products have traditionally been performed in the RVI model originally described by Eckstein et al. (25). This model involves daily intravaginal administration of the test article intravaginally to adult female New Zealand White rabbits for 10 days. The two main endpoints of this study are anatomic and histopathologic evaluations of the cervicovaginal mucosa. The RVI assay generates a composite irritation score based on an experienced pathologist's determination of the irritation severity measured against sham- or placebo-treated epithelia. Vaginal surface irritation is measured by gross anatomic observations of erythema and edema to generate a macroscopic MIS that gives equal weighting on a scale of 0 (normal) to 4 (severe) to visually observed pathologies. For the histopathologic MIS additional criteria, also scored scale of 0 to 4, are (*i*) characteristics of the epithelial lining (ulcerations or erosions), (*ii*) thickness of the submucosal layer (edema), (*iii*) leukocytic infiltration of the submucosal layer, and (*iv*) vascular injection (erythema). Again, each is given equal weight for generating a composite MIS to compare the test article's capacity to act as an irritant relative to an appropriate control. The determination of IL-8 levels induced by the various treatment regimens were determined by the collection of vaginal swab samples on days 4, 7, and 12, as well as a pretreatment swab taken on day 1. The swabs were washed in 1 mL of PBS. The amount of IL-8 present in each sample was determined by capture ELISA. The plates were visualized colorimetrically, and the amount of IL-8 was determined in duplicate by comparison with a standard curve of recombinant rabbit IL-8.

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