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IgG in cervicovaginal mucus traps HSV and prevents vaginal Herpes infections

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Abstract

IgG is the predominant immunoglobulin in cervicovaginal mucus (CVM), yet how IgG in mucus can protect against infections is not fully understood. IgG diffuses rapidly through cervical mucus, slowed only slightly by transient adhesive interactions with mucins. We hypothesize this almost unhindered diffusion allows IgG to accumulate rapidly on pathogen surfaces, and the resulting IgG array forms multiple weak adhesive crosslinks to mucus gel that effectively trap (immobilize) pathogens, preventing them from initiating infections. Here, we report herpes simplex virus serotype 1 (HSV-1) readily penetrated fresh, pH-neutralized *ex vivo* samples of CVM with low or no detectable levels of anti-HSV-1 IgG, but was trapped in samples with even modest levels of anti-HSV-1 IgG. In samples with little or no endogenous anti-HSV-1 IgG, addition of exogenous anti-HSV-1 IgG, affinity purified from intravenous immunoglobulin, trapped virions at concentrations below those needed for neutralization and with similar potency as endogenous IgG. Deglycosylating purified anti-HSV-1 IgG, or removing its Fc component, markedly reduced trapping potency. Finally, a *non*-neutralizing IgG against HSV-gG significantly protected mice against vaginal infection, and removing vaginal mucus by gentle lavage abolished protection.

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SUPPLEMENTARY MATERIAL

Supplementary Material is linked to the online version of the paper at <http://www.nature.com/mi>.

DISCLOSURE

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These observations suggest IgG-Fc has a glycan dependent “muco-trapping” effector function that may provide exceptionally potent protection at mucosal surfaces.

Keywords

mucosa; mucins; glycosylation; antibody; particle tracking; diffusion

INTRODUCTION

Large quantities of IgG are transported into female genital tract mucus secretions by the MHC class I-related neonatal Fc receptor,¹ resulting in at least ten-fold more IgG than IgA.² However, despite this predominance of IgG, the precise mechanism(s) by which secreted IgG can prevent vaginal infections are not well understood. Few studies have explored the potential protective role of IgG *within* the mucus secretions overlaying the epithelial tissue, which sexually transmitted viruses invariably encounter and must penetrate in order to reach target cells. Well-known Ab effector functions in blood and lymph (e.g., complement activation, opsonization, and ADCC) are absent or limited in healthy female genital secretions, which typically have little complement activity and few if any active leukocytes.³⁻⁵ These classical mechanisms of systemic immune protection also do not adequately account for the moderate but significant protection observed in the landmark Thai RV144 HIV vaccine trial.^{6, 7} The vaccination regimen modestly reduced the risk of HIV acquisition despite inducing primarily non-neutralizing Ab and otherwise offering little to no protection against systemic progression of infections once acquired, suggesting that protection likely occurred *prior* to initiation of infection. A better understanding of potential additional mechanisms of vaginal mucosal immunity will also likely be critical for developing effective vaccines against other sexually transmitted infections, including HSV, which has been shown to evade complement and other classical antibody-mediated protective mechanisms.⁸⁻¹⁰

Here, we seek to explore the hypothesis that secreted IgG may have evolved to work *in tandem* with mucus to trap and thereby exclude individual pathogens.³ Viruses must penetrate cervicovaginal mucus (CVM) to reach and infect their target cells in the vaginal epithelium; indeed, we have shown that HIV and human papillomavirus (HPV) are both capable of rapidly diffusing through human genital mucus secretions.^{11, 12} We also previously found that the diffusion of IgG (11 nm) was slowed slightly in human cervical mucus compared to in saline buffer, while much larger virus-like particles, including the capsids of Norovirus (38 nm) and HPV (55 nm), were not slowed by this mucus.¹² Thus, the slight retardation of the much smaller IgG molecules must be due to very transient (< 1 s), low-affinity bonds with the mucin mesh.¹² These observations prompt our hypothesis that, by making only transient low-affinity bonds with mucins, IgG is able to diffuse rapidly through mucus and accumulate on a pathogen surface. The array of Ab bound to the pathogen surface can in turn effectively trap the pathogen in mucus gel by ensuring at least some low-affinity bonds to the mucin mesh are present at any given time. Virions trapped in CVM cannot reach their target cells, and will instead be shed with post-coital discharge

and/or inactivated by spontaneous thermal degradation or other protective factors in mucus, such as defensins.^{13, 14}

RESULTS

Reduced HSV-1 mobility correlates with increasing endogenous anti-HSV-1 IgG in human CVM

We chose to explore this trapping-in-mucus hypothesis using HSV-1 (d ~180 nm), a highly prevalent sexually transmitted virus. We collected fresh, undiluted CVM obtained predominantly from donors with normal lactobacillus-dominated vaginal microbiota, as confirmed by Nugent scoring (Supplementary Table S1). HSV-1 virions expressing a VP22-GFP tegument protein construct, packaged at high copy numbers while maintaining native viral envelope integrity, were mixed into CVM pH-neutralized to mimic neutralization by alkaline seminal fluid. We then performed time-lapse microscopy of virion motions in real-time with high spatiotemporal resolution, and quantified virion mobility using multiple particle tracking over a long time scale. We observed substantial differences in HSV-1 mobility in CVM samples from different donors (Figure 1a and Supplementary Videos S1 and S2): in 7 of 12 CVM samples, most virions diffused distances spanning several microns over the course of 20 s, whereas in the remaining 5 CVM samples, the majority of virions were essentially trapped, moving less than their diameter (<200 nm) in 20 s.

Since IgG is the predominant immunoglobulin in human CVM,² we examined whether virion mobility correlated with endogenous virus-specific IgG in all 12 CVM samples measured using a whole-virus ELISA assay (Supplementary Table S2). In good agreement with our hypothesis, HSV-1 diffused rapidly through all CVM samples that had little or no detectable endogenous anti-HSV-1 IgG (< 0.2 µg/mL; detection limit 0.017 µg/mL) at rates only several-fold lower than their expected rates in water (Figure 1, Supplementary Figure S1, and Supplementary Table S3). In contrast, in samples with elevated levels of endogenous anti-HSV-1 IgG (≥ 0.6 µg/mL), most HSV-1 virions were effectively trapped. HSV-1 that was trapped in place over the first 20 s observation remained trapped in the same locations for at least 15 min (Supplementary Video S3 and Supplementary Figure S2). In the same CVM samples, control latex nanoparticles comparable in size to HSV-1 and engineered with muco-inert coatings (PS-PEG; d ~200 nm) exhibited rapid diffusion (Figure 1, Supplementary Video S4, and Supplementary Figure S1), in good agreement with our previous observations of the large pores present in human CVM (average ~340 nm).^{15, 16} Thus, the mucus mesh spacing was large enough for IgG-coated HSV-1 (at most 15-20 nm larger diameter even at saturation) to diffuse relatively unimpeded in the absence of adhesive interactions with mucin gel. Muco-adhesive latex nanoparticles of the same size (PS; d ~200 nm) were markedly slowed or immobilized in the same CVM secretions (Figure 1, Supplementary Video S5, and Supplementary Figure S1). Importantly, observations with PS-PEG and PS control particles confirmed that the general barrier properties of all samples, including those with low levels of endogenous anti-HSV-1 IgG, remained intact. After removal of ~90-95% of total IgG from these samples by dialysis at constant sample volume, HSV-1 became readily mobile (Supplementary Video S6 and Supplementary Figure S3), whereas PS beads remained immobilized (Supplementary Video S7 and Supplementary

Figure S3). HSV-1 mobility correlated only with endogenous HSV-1 specific IgG, and did not correlate with total IgG, IgA or IgM content (Supplementary Figure S4).

A well recognized mechanism of mucosal immune defense is ‘immune exclusion’ in which microorganisms in the gut are agglutinated by secreted polyvalent IgA and IgM into clusters too large to diffuse through mucus.^{17, 18} However, we observed little to no agglutinated HSV-1 in our experiments (see Supplementary Video S2 for example), consistent with previous findings that IgG is a relatively poor agglutinator.¹⁹ Together, these observations suggest that individual HSV-1 virions in samples with elevated endogenous levels of anti-HSV-1 IgG are slowed or trapped by multiple low-affinity bonds with CVM rather than by physical (steric) obstruction.

HSV-1 is trapped by sub-neutralizing levels of exogenously added anti-HSV-1 IgG

To confirm that trapping of HSV-1 in CVM was mediated specifically by IgG bound to virions and not by any other component in mucus that might be associated with elevated endogenous anti-HSV-1 IgG, we affinity purified HSV-1 specific IgG from human intravenous immunoglobulin (starting with a pure clinical IgG preparation), and mixed the purified IgG into CVM samples that had low endogenous anti-HSV-1 IgG. We found that addition of 1 µg/mL anti-HSV-1 IgG trapped HSV-1 with a potency comparable to that of endogenous anti-HSV-1 IgG (Figure 2, Supplementary Video S8, and Supplementary Figure S5; $p < 0.05$ compared to native specimen without addition of anti-HSV-1 IgG). We further tested lower anti-HSV-1 IgG doses (Supplementary Videos S9-11), and observed potent trapping of virions when ~333 ng/mL anti-HSV-1 IgG was added ($p < 0.05$), and partial trapping at 100 and 33 ng/mL anti-HSV-1 IgG added (both $p < 0.05$). As controls, muco-inert PS-PEG remained freely diffusive and muco-adhesive PS markedly slowed or immobilized in CVM samples treated with the highest anti-HSV-1 IgG doses (Supplementary Videos S12 and S13, respectively, and Supplementary Figure S6), confirming that the IgG did not cause HSV-1 trapping by altering mucus viscoelasticity or mesh spacing. Affinity-purified anti-HSV-1 IgG exhibited little neutralizing activity at 1 µg/mL and ~333 ng/mL (Figure 2b), based on reduction of plaque formation in Vero cells, suggesting that multiple low-affinity bonds between IgG and CVM can trap virions at IgG levels lower than those needed to neutralize. HSV-1 was also trapped by a humanized monoclonal anti-gD IgG in CVM (Supplementary Video S14) but not by control, non-specific IgG (Supplementary Video S15 and Supplementary Figure S7), underscoring the specificity of trapping via particular antibody-virus pairs, rather than a non-specific interaction or alteration of general mucus barrier properties. In good agreement with previous studies,^{12, 20} both polyclonal anti-HSV-1 IgG and monoclonal anti-gD IgG were only slightly slowed in CVM compared to saline (Supplementary Figure S8), suggesting both antibodies form only transient, low affinity bonds with CVM as individual molecules, yet facilitate effective trapping of virions once they accumulate on the viral surface by forming low-affinity but polyvalent IgG-mucin bonds.

IgG-mucus interaction is dependent on Fc-glycans

We next sought to determine the biochemical basis of the low-affinity bonds between IgG and CVM. The Fc domain of all IgGs harbors a conserved N-glycosylation site at Asn297,

and many IgG effector functions are Fc- and Asn297 glycan-dependent.²¹ Thus, we prepared F(ab')₂ fragments (Figure 3a and Supplementary Figure S9) and deglycosylated IgG (Figure 3b) from the same affinity-purified anti-HSV-1 IgG to minimize any changes in HSV-1 binding avidity (confirmed by ELISA), and measured the mobility of HSV-1 pre mixed with these modified analogs prior to addition to CVM (premixed to minimize interference by endogenous HSV-1 specific IgG). We found both F(ab')₂ and deglycosylated IgG exhibited substantially reduced trapping potency compared to intact IgG (Figure 3c; $p < 0.05$), suggesting that the low affinity bonds IgG forms with mucins are not only Fc-dependent, but also influenced by Fc glycosylation.

Non-neutralizing monoclonal IgG blocks vaginal HSV-2 infection in mice via IgG-mucus interactions

To determine whether trapping viruses in mucus can protect against infection *in vivo*, we evaluated the ability of a non-neutralizing monoclonal IgG₁ to reduce HSV-2 transmission in the pH neutral²² mouse vagina. This monoclonal IgG₁ binds to the relatively sparse gG surface glycoprotein, and exhibited no neutralization activity across all concentrations tested *in vitro* (Figure 4a); mouse IgG₁ also possesses little to no complement²³⁻²⁵ and ADCC^{26, 27} activity. We challenged mice vaginally with 2 ID₅₀ HSV-2 with and without anti-gG IgG₁, and assayed HSV infection by detection of virus shedding in vaginal lavages three days post inoculation, a more sensitive assay of infection than visual observation of lesions, viral isolation from sacral ganglia, or death.²⁸ Anti-gG IgG₁, at a concentration of 3.3 µg/mL and above, significantly protected against infection and reduced the average viral load compared to either medium alone or control, non specific IgG (Figure 4a and Supplementary Figure S10, $p < 0.05$). Interestingly, anti-gG IgG₁ appeared to only reduce the *rate* of successful vaginal HSV transmission; in mice that became infected, the extent of vaginal infection was comparable to that in mice receiving control IgG, suggesting that the anti-gG IgG dosed did not elicit effector functions that reduced the extent of virus spread in infected mice compared to control IgG (Supplementary Figure S10). We also evaluated protection in mice that received a gentle vaginal wash to remove mucus without detectable trauma to the epithelium (Figure 4b and Supplementary Figure S11). The removal of CVM increased susceptibility to HSV-2 in control mice from ~70% to ~100% but not the degree of HSV shedding in mice that became infected (Supplementary Figure S10). This moderate (~30%) increase in susceptibility is likely attributed to loss of innate protection by CVM itself: a CVM layer prevents immediate direct contact between viruses and the epithelium, and contains factors, such as defensins, that may further contribute to overall reduction infectious HSV flux to the epithelium. More importantly, removal of CVM completely abolished the ~50% extra protection (from ~70% to ~20% infection) afforded by anti-gG IgG₁ in native mice that cannot be attributed to innate immunity (Figure 4c). Consistent with the hypothesis that trapping in mucus may facilitate protection, these results together suggest much of the observed synergistic enhancement in protection by anti-gG IgG₁ when CVM is present most likely occurred *prior* to HSV reaching target cells, rather than by immune mechanisms that can facilitate protection at the cellular level (e.g., complement or ADCC). Our observations are also consistent with the poor complement and ADCC activity of mouse IgG₁, as well as numerous previous studies that have shown HSV can evade complement and other classical immune protective mechanisms.^{810, 29, 30} Since even a non-neutralizing

monoclonal IgG against a relatively sparse surface antigen can afford substantial protection, monoclonals against more abundant surface antigens, such as gD and gB, or those optimized to maximize interactions with mucus are likely to provide even more potent protection at mucosal surfaces *in vivo*.

DISCUSSION

The first evidence of antibody-mucin affinity can be traced back more than 30 years, when Kremer and Jager noted that infertility in humans is often caused by anti-sperm antibodies.^{31, 32} In cervical mucus samples with high levels of anti-sperm Ab, they found that both individual and agglutinated sperm make no forward progress and shake in place for hours until they die, despite vigorous flagellar motility. More recently, Phalipon *et al.* suggested secretory IgA can aggregate pathogenic *Shigella flexneri* in mouse nasal mucus secretions via the secretory component, anchoring the bacteria to the mucus gel and thereby 'excluding' them from infectious entry.³³ In both of the above instances, the authors assumed that the antibodies were attached firmly to the mucins. However, FRAP experiments by Olmsted *et al.*,¹² and those here, clearly demonstrate that IgG and other Ab diffuse rapidly in human genital mucus, slowed only slightly and transiently compared to their diffusion in water, and with no non-recoverable fraction indicative of permanently immobilized Ab. Using a complementary technique called fluorescence imaging of profiles (FIP), Saltzman *et al.* also showed similar rapid Ab diffusion in human cervical mucus.²⁰ Such rapid diffusion of individual Ab molecules in mucus can only be explained by weak and short-lived adhesive interactions between Ab and the mucin mesh. Thus, trapping of individual sperm or pathogens by specific Ab in mucus gel most likely reflects multiple weak interactions between mucins and an array of bound Ab. These results contrast sharply with recent work by Fahrbach *et al.* that suggested endogenous IgG binds tightly to mucins.³⁴ This discrepancy is likely due to experimental artifacts from the use of fluorescently-tagged Ab to label endogenous Ab in the latter work, which would create large immune complexes that exhibit far slower diffusivity than individual Ab molecules, consequently misinterpreted as high affinity binding of individual Ab molecules to mucins.

The mucin-like Fc γ binding protein (Fc γ BP) has also been proposed to serve an immunological role in mucus through its ability to bind strongly to IgG-Fc.³⁵ Nevertheless, more recent evidence indicates the primary function of Fc γ BP is instead to stabilize gastrointestinal mucus gel by covalently cross-linking Muc2 mucins.^{36, 37} An IgG Fc-Fc γ BP mucin crosslinking mechanism that relies on strong binding of IgG by Fc γ BP also directly contradicts numerous prior efforts that have failed to detect any significant binding of individual Ab to mucins.^{3, 12, 20, 38, 39} Finally, Fc γ BP binds broadly to all IgG, and the interaction is thus subject to competitive inhibition.⁴⁰ In our experiments where exogenous HSV-1 specific IgG was added to CVM, total levels of IgG already present in the samples were hundreds- to thousands-fold higher than the HSV-1 specific IgG doses added. Thus, for Fc γ BP to be responsible for the observed trapping phenomena, Fc γ BP must have been present in greater molar quantities than native IgG, which is unlikely given that the protein has not been routinely identified in proteomic screens of human genital tract fluid (see Andersch-Bjorkman *et al.*⁴¹ vs. others⁴²⁻⁴⁴). In contrast to previous studies, by examining the effect of IgG on virions in mucus gel rather than probing directly for interactions

between individual IgG molecules and mucins, we were able to document not only the potent trapping of individual virions by multiple surface-bound IgG (Figure 5), but also that the IgG-mucin interactions are Fc- and glycan-dependent.

We have previously shown that HIV-1 and HSV-1 can be effectively immobilized in acidic, lactic acid-rich CVM (at least 1000 fold reduced D_{eff} compared to in buffer).^{11, 16} However, both virions readily penetrate more pH-neutral mucus, similar to mucus secretions exposed to alkaline semen or depleted of lactobacilli, such as in women with bacterial vaginosis.⁴⁵ The ability for IgG-mucin interactions to trap virions (~1500 fold reduced D_{eff} compared to in buffer) may therefore provide a crucial mechanism to effectively reinforce the mucus barrier. The extent of reduction in virus mobility observed here contrasts sharply with observations by Shukair *et al.*,⁴⁶ who reported an activity in genital mucus specimens from HIV negative women (i.e., free of virus-specific IgG) that hindered HIV mobility by only a few-fold on average. The mucus layer coating the female reproductive tract may be only ~50-100 μm thick, and readily penetrated by virions within minutes even if they are slowed a few fold in mucus compared to in buffer.¹¹ Thus, to effectively reduce the flux of virions reaching target cells, it is likely essential for virions to be slowed by orders of magnitude.

Trapping virions in genital tract mucus should markedly reduce heterosexual transmission of viral infections. Women acquire many of the major sexually transmitted viral infections (e.g., HIV, HPV, and HSV) at rates on the order of 1 per 100 to 1000 sex acts on average (see Supplementary Text). This suggests few if any of these virions are able to infect target cells per intercourse, which reflects a combination of the diffusional and other innate barrier properties of genital secretions. Therefore any reduction in the flux of virions that reach target cells should proportionally reduce transmission rates. Indeed, the effectiveness of the IgG-mucin interactions reported here may be reflected in part by the clinical observation that HSV vaginitis is generally less common than HSV infection of the anogenital skin (vulva and groin), which are not protected by genital mucus secretions. Blocking initial infection altogether, rather than attempting to clear initial infections, may be especially critical for infections that are difficult, if not impossible, to cure once established (e.g., HSV, HIV). Our findings not only underscore the importance of monitoring both mucosal and systemic immune responses, but also motivate developing vaccines that elicit sufficient secreted Ab to trap pathogens in mucus in addition to eliciting neutralizing titers of systemic Ab. In the recent gD2-AS04 HSV vaccine trial,^{47, 48} protective efficacy was initially observed in seronegative women but not in men or seropositive women, and a larger study of seronegative women revealed only moderate efficacy against HSV-1 (~35% efficacy against HSV-1 infection and 58% efficacy against HSV-1 genital disease) but interestingly no protection against HSV-2. In both studies, the vaccine elicited neutralizing serum Ab against HSV as well as HSV specific cellular immune responses in all women and men. However, because *mucosal* levels of Ab were not monitored, it remains unclear whether what little protection was observed could have correlated with mucosal Ab response. It is likely that generating sufficient mucosal Ab levels remains a major bottleneck to an effective HSV vaccine. Inducing secreted Ab that bind to 'non-neutralizing' surface epitopes should trap pathogens as effectively as those that bind to neutralizing epitopes, a prospect that may

broaden potential antigen targets for vaccine development, especially against virions with rapidly evolving neutralizing epitopes.

Fc-mediated trapping of pathogens in mucus, which directly blocks infections at the portals of entry, may represent an exceptionally potent mechanism by which the immune system can rapidly adapt and reinforce multiple mucosal surfaces against diverse and rapidly evolving pathogens. In pilot studies, we found LPS-specific monoclonal IgG, but not control IgG, immobilized individual *Salmonella typhimurium* in mucus secretions lining mouse gastrointestinal tract tissues without inhibiting the flagella beating apparatus and without causing aggregation, i.e., independent of the classical, aggregation based mechanism of immune exclusion (data not shown; Subramani, Lai). The observed trapping in both CVM (predominantly Muc5b mucins) and gastrointestinal mucus (Muc2 mucins) suggests the molecular basis for Fc-mucin affinity may be common among major secreted mucins – the long densely glycosylated fibers that form mucus gels – and possibly mediated by glycans, since sugars represent the major constituent of mucins (up to 80% by dry weight⁴⁹). Nevertheless, significant technical challenges remain in elucidating the precise entities on mucins responsible for the observed Fc mucin affinity because biochemical perturbations of the mucus gel typically destroy the native mucin mesh microstructure and gel viscoelasticity⁵⁰ needed to trap pathogens in the gel. Improved understanding of the molecular basis of Fc-mucin affinity will likely offer critical insight into understanding and identifying subpopulations with greater susceptibility to infection, as well as therapeutic strategies to enhance this mucosal immune protective mechanism.

MATERIALS AND METHODS

Culture and purification of fluorescent HSV-1

The HSV-1 mutant 166v,⁵¹ encoding a VP22-Green Fluorescent Protein (GFP) tegument protein packaged into HSV-1 at relatively high copy numbers,⁵² was kindly provided by Richard Courtney and used in all microscopy and ELISA studies, besides those for *in vivo* experiments. The addition of GFP to the VP22 protein appears to have no deleterious effects on viral replication,⁵¹ and the fluorescence of 166v was consistently more intense than that of HSV-1 mutants encoding other GFP fusion proteins. 166v was expanded at an MOI of 3 on confluent monolayers of HaCat cells maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 5% FBS, 1× L-glutamine and 1× Penicillin/Streptomycin. Culture medium was collected 16-18 hr post infection and twice centrifuged at 1000 ×g for 5 min to remove cell debris. The resulting supernatant was split into 30 mL aliquots and precipitated overnight with a polyethylene glycol (PEG)/salt solution. Briefly, 10 mL of 1.55 M NaCl was added to 30 mL of crude virus supernatant, followed by 10 mL of 40% PEG 8000 (Sigma, St. Louis, MO). After an overnight incubation at 4°C the virus/PEG solution was centrifuged at 2555 ×g and 4°C for 1 hr. The virus pellet was then resuspended in 1× PBS and centrifuged through a continuous 20-50% (w/w) sucrose in PBS gradient for 1 hr at 74,119 ×g. The resulting virus band was collected, diluted 1:5 in PBS, layered over 30% (w/w) sucrose in PBS, and centrifuged for 1.5 hr at 83,472 ×g to pellet virus for further purification. Purified virus pellet was resuspended in PBS and stored as single use aliquots at –80°C.

Cervicovaginal mucus (CVM) collection and characterization

CVM collection was performed as published previously.^{11, 16} Briefly, undiluted CVM secretions, averaging 0.3 g per sample, were obtained from women of reproductive age, ranging from 20 to 32 years old (27.4 ± 0.9 years, mean \pm SEM), by using a self sampling menstrual collection device following protocols approved by the Institutional Review Board of the University of North Carolina – Chapel Hill. Informed consent of participants was obtained after the nature and possible consequences of the study were explained. Participants inserted the device into the vagina for at least 30 s, removed it, and placed it into a 50 mL centrifuge tube. Samples were centrifuged at $230 \times g$ for 2 min to collect the secretions. Aliquots of CVM for lactic acid and Ab measurements (diluted 1:5 with $1 \times$ PBS and stored at -80°C) and slides for gram staining were prepared immediately, and the remainder of the sample was stored at 4°C until microscopy, typically within a few hours. Samples were collected at random times throughout the menstrual cycle, and cycle phase was estimated based on the last menstrual period date normalized to a 28 day cycle. No samples were ovulatory based on visual observation (none exhibited spinnbarkeit). Samples that were non uniform in color or consistency were discarded. Donors stated they had not used vaginal products nor participated in unprotected intercourse within 3 days prior to donating. All samples had $\text{pH} < 4.5$; none had bacterial vaginosis (BV) based on Gram staining and Nugent scoring, following scoring criteria described previously.⁵³ For lactic acid and Ab measurements, CVM aliquots were thawed and centrifuged for 2 min at $21,130 \times g$ to obtain cell free supernatant containing lactic acid and Ab. Lactic acid content was measured using a D-/L-lactic acid kit (R Biopharm, Darmstadt, Germany) according to manufacturer protocol, but adapted to a 96-well format. Methods for Ab measurements are detailed in the Supplementary Materials and Methods.

Preparation and characterization of anti-HSV-1 Ab

Anti-HSV-1 IgG was affinity purified from intravenous immunoglobulin, and used to prepare anti-HSV-1 F(ab')_2 and deglycosylated anti-HSV-1 IgG. Detailed methods are provided in the Supplementary Materials and Methods.

Neutralization assay

Purified HSV-1 (~550 PFU; $5 \mu\text{L}$) was incubated with $95 \mu\text{L}$ of HSV-1 specific IgG solution at different final concentrations for 1 hr with end over end mixing. The mixture was then diluted with $210 \mu\text{L}$ of media, of which duplicate $150 \mu\text{L}$ aliquots were transferred to confluent Vero cell monolayers in a 6 well plate. Plates were incubated at 37°C for 1 hr with periodic rocking to ensure that the plates did not dry out, before the HSV-1/Ab mixture was aspirated off and wells were washed with 2 mL of PBS. The plates were then incubated for 3 days at 37°C in 2% carboxymethyl cellulose in EMEM supplemented with $1 \times$ L-glutamine and $1 \times$ Penicillin/Streptomycin, before staining with 1% crystal violet solution, and the resulting plaques were manually counted and compared to control wells to determine the extent of neutralization.

Fluorescence recovery after photobleaching (FRAP)

FRAP was performed following methods similar to those described by Olmsted *et al.* FITC-labeled Ab was added to mucus or saline at 10% v/v and gently stirred to achieve uniform distribution. FRAP experiments were conducted using an LSM 510 confocal microscope, with a roughly $40\ \mu\text{m} \times 40\ \mu\text{m}$ ROI bleached with 405/488 lasers at 100% laser intensity over ~ 3 sec. For mucus samples, ROIs were chosen to minimize epithelial cells within the bleached volume. After bleaching, the fluorescence intensity of Ab was monitored over time with background subtracted out and values adjusted for photobleaching of the entire image over time based on control ROIs.

Multiple particle tracking of HSV-1 in CVM

To mimic neutralization of CVM by alkaline seminal fluid, we titrated CVM to pH 6.8-7.1 using small volumes ($\sim 3\%$ v/v) of 3 N NaOH, and confirmed pH using a micro pH electrode (Microelectrodes, Inc., Bedford, NH) calibrated to pH 4, 7 and 10 buffers. Samples were either untreated or treated by addition of known amounts of purified anti-HSV-1 IgG or control (anti-biotin) IgG. Control beads consisted of red or green fluorescent 200 nm carboxyl-modified polystyrene particles (Molecular Probes, Eugene, OR), either uncoated (PS; muco-adhesive) or covalently conjugated with low molecular weight (2 kDa), amine-functionalized polyethylene glycol (PEG; Rapp Polymere, Tuebingen, Germany) to produce coated particles (PS-PEG; muco-inert), as previously described.¹⁵ Fluorescent virions or control beads (approximately 10^8 – 10^9 particles/mL) were added at 5% v/v to 20 μL of CVM placed in a custom made glass chamber, and incubated for 1 hr at 37°C prior to microscopy. The translational motions of the particles were recorded using an EMCCD camera (Evolve 512; Photometrics, Tucson, AZ) mounted on an inverted epifluorescence microscope (AxioObserver D1; Zeiss, Thornwood, NY), equipped with an Alpha Plan Apo 100x/1.46 NA objective, environmental (temperature and CO₂) control chamber and an LED light source (Lumencor Light Engine DAPI/GFP/543/623/690). Videos (512×512 , 16-bit image depth) were captured with MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) at a temporal resolution of 66.7 ms and spatial resolution of 10 nm (nominal pixel resolution 0.156 $\mu\text{m}/\text{pixel}$) for 20 s. The tracking resolution was determined by tracking the displacements of particles immobilized with a strong adhesive, following a previously described method.⁵⁴ Particle trajectories were analyzed using MetaMorph software as described previously;^{11, 15, 16} image contrast was adjusted to improve particle visibility, but the same contrast level was applied throughout the entire video and did not bias toward any particle population. Sub-pixel tracking resolution is obtained by determining the precise location of the particle centroid by light-intensity-weighted averaging of neighboring pixels. Trapped particles were defined as those with effective diffusivity ($D_{\text{eff}} < 0.01\ \mu\text{m}^2/\text{s}$ at a time scale (τ) of 1 s (i.e., particles move less than their diameter within 1 s). In a subset of experiments, we confirmed that particles defined as trapped over the course of 20 s based on this criterion remain confined to the same locations over more than 15 min. The slope α of the log-log mean square displacement ($\langle \text{MSD} \rangle$) vs. time scale plot provides a further measure of particle mobility: $\alpha = 1$ for pure unobstructed Brownian diffusion, e.g., particles in water, α becomes smaller as obstruction to particle diffusion increases, and α is zero for permanently trapped particles. At least five independent experiments in CVM from different

donors, with $n = 100$ particles per experiment, were performed for each condition. For a subset of donors, similar observations were made at least twice in samples obtained on separate days to ensure reproducibility, but only one sample was used for analysis.

Mouse vaginal HSV-2 challenge model

All experiments conducted with mice were performed in accordance with protocols approved by the Johns Hopkins University Animal Care and Use Committee satisfying the requirements of the E.E.C. Guidelines (1986) and U.S. Federal Guidelines (1985). Female CF-1 mice (6-8 weeks old; Harlan, Frederick, MD) were treated with Depo-Provera® (medroxyprogesterone acetate, 2.5 mg/mouse) by subcutaneous injection into the right flank 6-8 days prior to use. Depo-Provera® synchronizes mice in a prolonged diestrus like state, in which the vaginal epithelium thins and susceptibility of the tissue to infection increases.⁵⁵ Depo Provera®-treated mice were randomly divided into groups of ten. The mouse vagina is pH neutral;²² therefore, we did not attempt to modify vaginal pH prior to inoculation. Inocula were prepared by mixing HSV-2 (final dose 2 ID₅₀; strain G, ATCC, Manassas, VA) with medium or different concentrations of control (anti-biotin) or anti-gG IgG (8.F. 141; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and incubating for 1 hr at 37°C. Mice were inoculated with 20 µL of HSV-2 solution, delivered to the vagina using a 50 KL Wiretrol (Drummond, Broomall, PA), fire-polished to avoid damage to the vaginal epithelium. In some studies, the mouse vagina was gently washed with ~10 mL of normal saline delivered at 1 mL/min through a smooth ball-tipped gavage needle connected to a syringe pump, prior to HSV-2 challenge. Removal of mucus by this process was measured using a fluorimetric mucin assay, as previously described.⁵⁶ Importantly, the gentle wash did not damage the vaginal epithelium, as confirmed by microscopy with a fluorescence-based dead cell stain (YOYO-1) that assesses membrane integrity,⁵⁵ compared to conventional lavage and/or vaginal swabbing with a cotton tip, which induced significant epithelial damage. YOYO-1 has been previously used to reveal toxicity caused by detergent-based microbicides that led to increased susceptibility to HSV infection.⁵⁵ Tissue sectioning and H&E staining was performed by the Animal Histopathology lab at the University of North Carolina – Chapel Hill. Infection was assayed three days post-inoculation by detection of virus in vaginal lavages. Briefly, 50 µL of medium was pipetted in and out of the vagina 20 times, diluted to 0.2 mL and placed on target cells (ELVIS® HSV Test System; Diagnostic Hybrids, Athens, OH); infected cells (foci) were identified one day later, following manufacturer protocol. Scores for virus shedding were assigned on a scale of 0-4 based on the approximate density of foci observed (“0”: 0; “0.5”: <100; “1”: 100 500; “2”: 500 1000; “3”: 1000+; “4”: saturated). At least three independent experiments were performed for each condition, with $n = 10$ animals per experimental group ($n = 30$ total).

Statistical analysis

Correlation between endogenous anti-HSV-1 IgG levels and average particle or virus D_{eff} in individual CVM samples was measured using Pearson’s correlation coefficient (r). Statistical comparisons were limited to two groups (test group compared with the appropriate control group performed during the same experiment). Fisher’s exact test was used to determine the statistical significance of reductions in % mice infected. A two tailed Student’s t test (paired for comparisons of Ab-treated vs. native CVM for the same CVM

samples) was used for all other comparisons. Differences were deemed significant at an alpha level of 0.05. All values are reported as mean \pm SEM unless otherwise indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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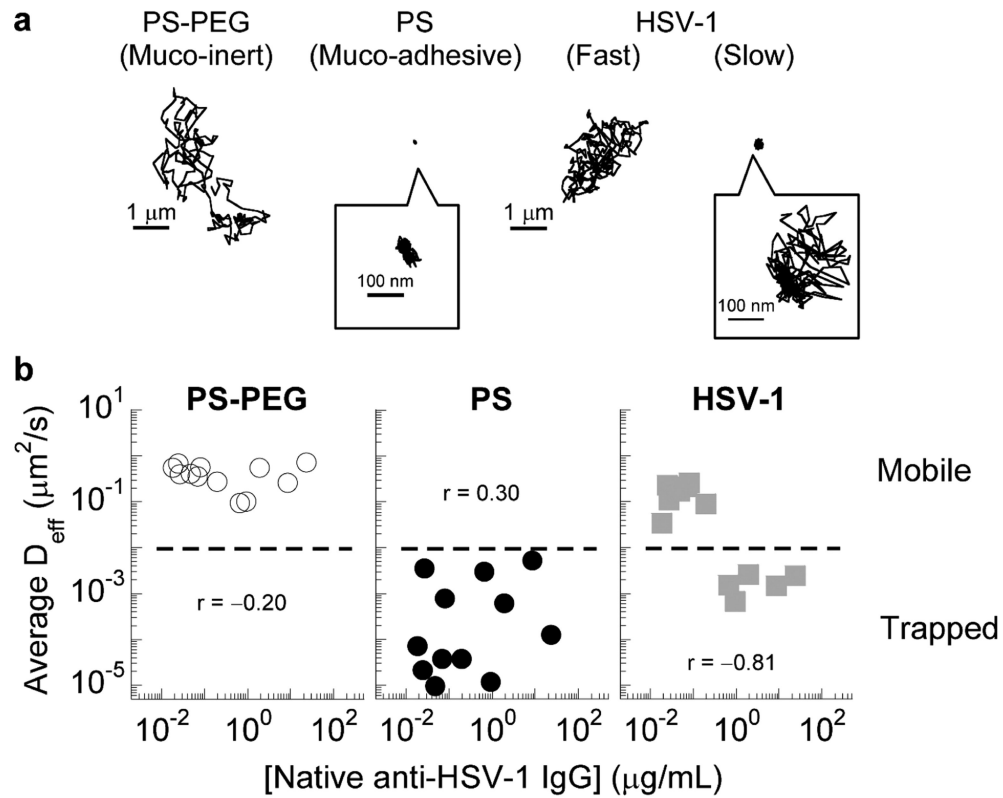
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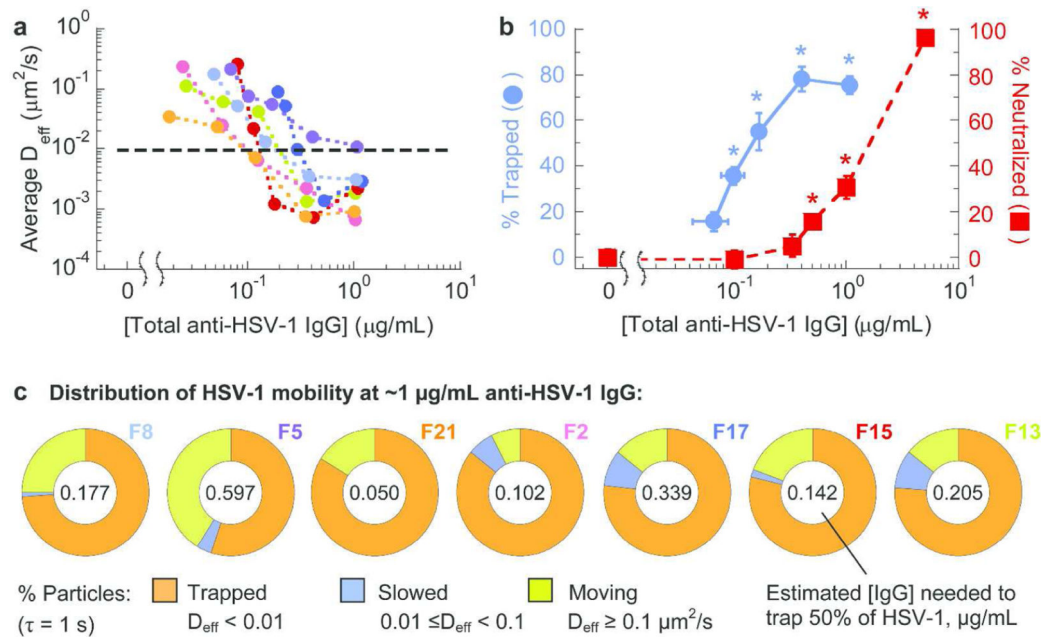
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127x101mm (1200 x 1200 DPI)

Figure 1.

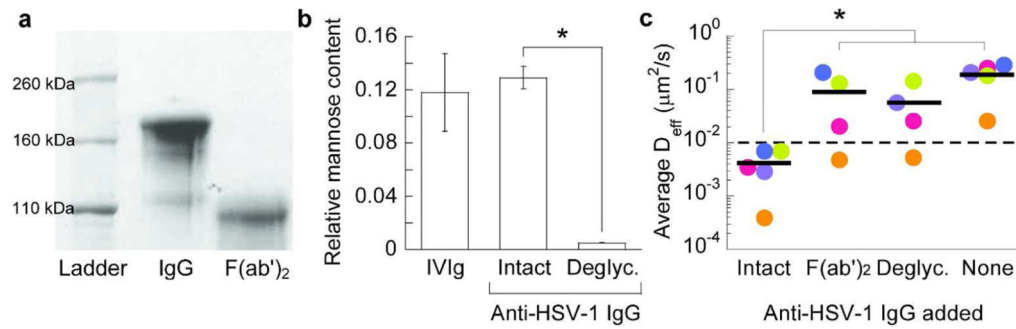
HSV-1 is immobilized in cervicovaginal mucus (CVM) samples with elevated endogenous anti-HSV-1 IgG but readily mobile in samples with low endogenous anti-HSV-1 IgG. Fluorescent HSV-1 or control particles were added to CVM, and their motions were analyzed by multiple particle tracking methods. **(a)** Representative 20 s traces of HSV-1 ($d \sim 180$ nm) and control particles ($d \sim 200$ nm) with effective diffusivity (D_{eff}) at a time scale τ of 1 s within one SEM of the mean. Control particles include muco inert (PEG coated; PS PEG) and muco adhesive (uncoated; PS) polystyrene beads, which are freely diffusive and trapped in human CVM, respectively, as previously shown.¹⁵ **(b)** Geometric average D_{eff} ($\tau = 1$ s) for PS-PEG, PS and HSV-1 in individual CVM samples from unique donors ($n = 12$, each experiment performed independently) as a function of endogenous anti-HSV-1 IgG. Dashed lines represent the D_{eff} cutoff below which particles are permanently trapped (moving less than their diameter within 1 s). Pearson's correlation coefficients (r) are indicated.



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Figure 2.

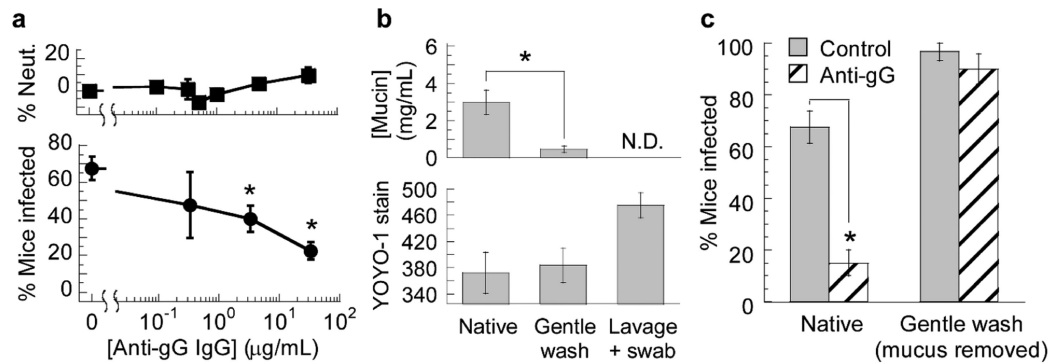
Anti-HSV-1 polyclonal human IgG added to cervicovaginal mucus (CVM) samples with low endogenous anti-HSV-1 IgG potently traps HSV-1. HSV-1 mobility was quantified in aliquots of the same CVM samples with different amounts of anti-HSV-1 IgG added. **(a)** Comparison of effective diffusivity (D_{eff} ; $\tau = 1 \text{ s}$) for HSV-1 in CVM samples ($n = 7$, each experiment performed independently) with different amounts of total anti-HSV-1 IgG (sum of endogenous and added IgG). Different colored circles represent distinct samples. **(b)** *In vitro* neutralization vs. trapping potency of anti-HSV-1 IgG. Neutralization was assayed based on reduction of HSV plaque formation in Vero cells; trapping was defined as D_{eff} ($\tau = 1 \text{ s}$) below $0.01 \mu\text{m}^2/\text{s}$. See Materials and Methods for additional details. Total IgG was averaged across samples for each treatment group. Error bars represent SEM. * indicates statistically significant difference compared to respective controls ($p < 0.05$). **(c)** Distribution of particle speeds in samples treated with $1 \mu\text{g}/\text{mL}$ IgG (annulus chart), and estimated concentration of total IgG ($\mu\text{g}/\text{mL}$) needed for 50% trapping (number in center). Donor ID is indicated for each sample with colors matching those in **(a)**.



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Figure 3.

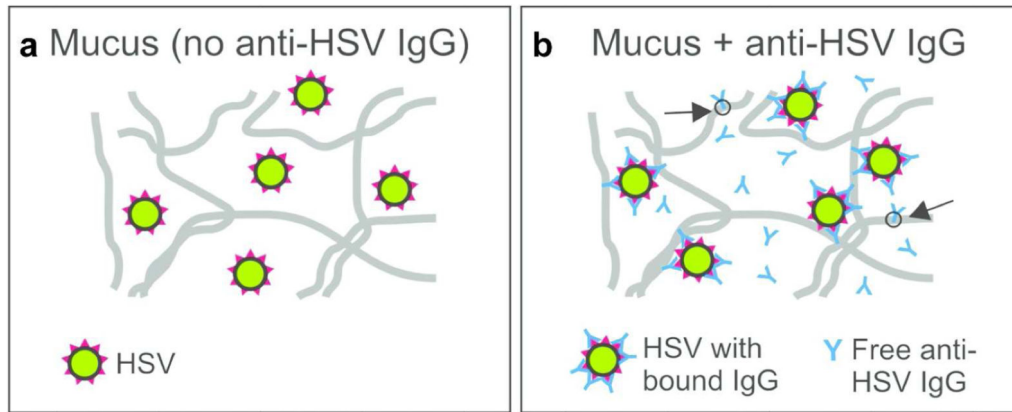
IgG mucin affinity is Fc and glycosylation dependent. **(a)** Preparation of anti-HSV-1 F(ab')₂ confirmed by SDS PAGE; full length gel is presented in Supplementary Figure S9. **(b)** Preparation of deglycosylated anti-HSV-1 IgG confirmed by lectin-binding assay (absorbance of IgG bound ConA normalized to amount of IgG). Error bars represent SEM. **(c)** Mobility (D_{eff} ; $\tau = 1$ s) of HSV-1 in cervicovaginal mucus (CVM) with low endogenous anti-HSV-1 IgG incubated with various HSV-1 specific Ab: 1 Kg/mL affinity purified native IgG (“Intact”), 667 ng/mL F(ab')₂, and 1 g/mL deglycosylated IgG compared to HSV-1 in native CVM (“None”). Distinct samples ($n = 4$ – 5 , each experiment performed independently) are indicated with different color circles; averages are indicated by solid lines. Dashed line represents the D_{eff} cutoff below which particles are permanently trapped (moving less than their diameter within 1 s). * indicates statistically significant difference ($p < 0.05$).



168x60mm (1200 x 1200 DPI)

Figure 4.

A non-neutralizing monoclonal IgG against the gG epitope protects against vaginal HSV-2 infection in mice via IgG-mucin interactions. **(a)** In vivo protection vs. in vitro neutralization (% Neut.) of HSV-2. Neutralization was assayed based on reduction of HSV plaque formation in Vero cells. Depo-Provera®-treated mice were inoculated with HSV-2 mixed with control or anti-gG IgG. Infection was assayed three days post inoculation by detection of virus in vaginal lavages using the ELVIS® HSV Test System. **(b)** Mucin concentration in vaginal fluid collected from the native or gently washed mouse vagina, and YOYO-1 staining for vaginal epithelial cell damage in gently washed or conventionally lavaged and swabbed (cotton tip) mice. N.D. = no data. **(c)** *In vivo* protection by 33 μg/mL anti-gG IgG is lost when mouse CVM is removed by gentle washing using a syringe pump. Data represent at least three independent experiments, each with n = 10 mice per group (*in vivo*; total n = 40 per group for data in **(a)**, n = 30 for **(c)**) or performed in triplicate (*in vitro*). Error bars represent SEM. * indicates statistically significant difference compared to control ($p < 0.05$).



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Figure 5.

Proposed mechanism of Ab-mediated trapping of viruses in mucus. Schematic showing (a) HSV readily penetrates native CVM with little to no endogenous anti-HSV IgG, and (b) anti-HSV IgG traps HSV in CVM by multiple transient, low affinity bonds with mucins. By forming only short-lived, low affinity bonds with mucus, free Ab, such as IgG, are able to diffuse rapidly through mucus and bind to viruses. As IgG molecules accumulate on the virus surface, they form multiple low-affinity bonds between the virus and mucus gel. A sufficient number of these transient low-affinity bonds ensure viruses are effectively trapped in mucus at any given time, thereby reducing the flux of infectious virions that can reach target cells. In a similar fashion, IgM, with its 10 relatively low-affinity Fab components, is well known to make essentially permanent polyvalent ‘high avidity’ bonds. Arrows indicate the small fraction of free (not virus-bound) IgG (~10-20%) that will interact with mucins at any moment in time^{3, 12}.