Diffusion of Macromolecules and Virus-Like Particles in Human Cervical Mucus

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ABSTRACT To determine whether or not large macromolecules and viruses can diffuse through mucus, we observed the motion of proteins, microspheres, and viruses in fresh samples of human cervical mucus using fluorescent recovery after photobleaching and multiple image photography. Two capsid virus-like particles, human papilloma virus (55 nm, ~20,000 kDa) and Norwalk virus (38 nm, ~10,000 kDa), as well as most of the globular proteins tested (15–650 kDa) diffused as rapidly in mucus as in saline. Electron microscopy of cervical mucus confirmed that the mesh spacing between mucin fibers is large enough (20–200 nm) for small viruses to diffuse essentially unhindered through mucus. In contrast, herpes simplex virus (180 nm) colocalized with strands of thick mucus, suggesting that herpes simplex virus, unlike the capsid virus particles, makes low-affinity bonds with mucins. Polystyrene microspheres (59–1000 nm) bound more tightly to mucins, bundling them into thick cables. Although immunoglobulins are too small to be slowed by the mesh spacing between mucins, diffusion by IgM was slowed by mucus. Diffusion by IgM-$Fc_{5\mu}$, the Fc pentamer core of an IgM with all 10 Fab moieties removed, was comparably slowed by mucus. This suggests that the Fc moieties of antibodies make low-affinity bonds with mucins.

INTRODUCTION

Mucus that coats the surfaces of the gastrointestinal, respiratory, and reproductive tracts provides the outermost barrier against pathogens. Secreted mucins, the gel-forming component of mucus, are large proteins (10–40 MDa, 0.5–5 μm long) that are highly glycosylated (up to 80% sugar by weight) and that form an entangled viscoelastic gel (Carlstedt and Sheehan, 1989; Cone, 1999; Forstner et al., 1995; Nieuw Amerongen et al., 1998).

Early work indicated that mucus slows the diffusion of small molecules and protons (Allen, 1978, 1981; Desai et al., 1992; Desai and Vadgama, 1991; Williams and Turnberg, 1980; Winne and Verheyen, 1990). This led to the belief that large proteins would not penetrate mucus gels. These early experiments measured the diffusional flux of particles between two stirred chambers through a layer of mucus sandwiched between filters. Ostensibly straightforward, this method is subject to major artifacts (Sanders et al., 2000), including an inability to control, or adequately account for, the unstirred layer of fluid adherent to the outer surfaces of the filters. Moreover, mucus is very sticky and can clog filters, reducing the apparent diffusion coefficient.

Over the past several years, two techniques have been developed for directly measuring diffusion of molecules in fresh samples of mucus subjected to minimal dilution and handling: fluorescent recovery after photobleaching (FRAP) and fluorescent imaging of profiles. In these methods, the diffusion of fluorescently labeled particles can be observed without the use of filters or need for stirring. These methods have shown that many proteins (1.2–150 kDa), including immunoglobulin (IgG), diffuse in mucus nearly as fast as they diffuse in water, indicating that the mesh spacing between fibers of mucins must be large enough not slow their diffusion (Henry et al., 1992; Radomsky et al., 1990; Saltzman et al., 1994; Zeitlin et al., 1998).

Few studies have looked at diffusion of larger particles in mucus. In vivo experiments have explored uptake of microspheres in the gastrointestinal tract of rats. Small polystyrene microspheres (14 and 415 nm) placed in the distal colon of rats, were later observed adjacent to the mucosa whereas larger microspheres (1.1 μm) remained in the lumen and in the mucus layer of the colon (Szentkuti, 1997). Others have reported that microspheres (50 nm, 500 nm, and 1 μm) fed orally to rats were taken up by Peyer patches in the gastrointestinal tract, but also showed that microspheres were trapped by the mucus layer around the Peyer patches (Jani et al., 1992). However, these studies were not able to differentiate between microspheres diffusing through mucus and microspheres being transported or moving because of peristaltic stirring. With this in mind, one aim of the present study was to determine whether proteins larger than IgG (150 kDa), and even viruses, can diffuse in mucus.

Using FRAP and fluorescent imaging of profiles, Saltzman et al. (1994) reported that Igs can diffuse rapidly through mucus, although not as rapidly as in saline. Their results did not clarify whether the Igs were slowed by the

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mesh spacing between mucin fibers, or whether antibodies bind with low affinity to mucins. Kremer and Jager (1976) found that antisperm antibodies, including IgG, IgA, and IgM, were found to be capable of cross-linking motile sperm to the mucus gel, causing the sperm to shake in place. This "shaking phenomenon" clearly suggested that antibodies bound to sperm cross-link the sperm to the mucus gel, trapping the sperm in the gel by forming multiple low-affinity bonds with the mucin fibers. Thus, another aim of this study was to determine whether antibodies are retarded by the mesh spacing and/or by low-affinity bonds with mucins. Also, we sought to determine which antibody moiety forms low-affinity bonds with mucins.

That large proteins are small enough to diffuse unhindered by the mucin fiber mesh spacing was suggested by previous electron microscopy (EM) of mucus gels. Early electron micrographs of mucus indicated that the mesh spacing was 90–3000 nm (Chretien et al., 1975; Poon and McCoshen, 1985; Yudin et al., 1989), much larger than the hydrodynamic diameter of most globular proteins (2–15 nm). But these same micrographs indicated that the diameter of the mucin fibers was ~50 nm, much too large to be consistent with the biochemical structure of mucin fibers, or the thickness of individual mucin fibers as observed by EM and by atomic force microscopy (AFM). Biochemical analysis (Yurewicz et al., 1987) of the sugars attached to mucins predicts, and EM (Slayter et al., 1991) and AFM (McMaster et al., 1999) of purified individual mucins adhering to a substrate surface all indicate that the diameter of mucin fibers is 3–10 nm. Thus, the much larger fibers observed in earlier EM of mucus gels was probably because of fibers of mucins becoming bundled together during the preparative and fixation process. Such bundling, or cabling, of mucin fibers not only artifactually increases the apparent fiber diameter, it also artifactually increases the apparent mesh spacing between mucin fibers. Therefore, the final aim of this study was to obtain transmission electron micrographs of human cervical mucus (HCM) gels by a novel fixation method that seems to minimize artifactual thickening of mucin fibers and, hence, more likely reveals the actual mesh spacing.

MATERIALS AND METHODS

Mucus collection

HCM samples were collected using procedures approved by the Review Board on the Use of Human Subjects at the Johns Hopkins University and UC-Davis School of Medicine. Women not on hormonal contraceptives monitored their hormonal cycle to determine their time of ovulation. Cervical mucus at the time of ovulation, or midcycle, has lower viscosity and is produced in greater volume than the mucus produced during the rest of the cycle, and is therefore more easily sampled. At midcycle, women came to the health center and their mucus was collected from the cervix with a sterile 1-cc syringe (no needle). Samples were immediately delivered to the laboratory where they were stored at 4°C until used.

Only those samples that met criteria outlined by the World Health Organization for sperm-cervical mucus interaction were used (WHO, 1992). The samples were weighed, clarity was observed visually, and pH was measured using a glass microelectrode (MI-414–6 cm, Microelectrodes, Inc., Bedford, NH). Spinnbarkeit was measured by placing a small sample of mucus on a glass slide. Then a wooden cytology spatula (Baxter Healthcare Corp., McGaw Park, IL) was pressed against the sample and slowly lifted vertically. Spinnbarkeit is the length at which the mucus thread breaks. Samples accepted for further analysis were $\geq$0.2 ml in volume, free of cellular debris, in the pH range 6.4–8.0, and had spinnbarkeit $\geq$5 cm. Samples were used within one week of receiving them.

Fluorescently labeled proteins, viruses, and microspheres

Fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, OR)-labeled purified human IgA, IgG, IgM, IgM-Fab$_\mu$ (the Fab fragment of an IgM), IgM-Fc$_{5\mu}$ (the Fc pentamer core of an IgM), and aggregated secretory IgA (sIgA) were purchased from Jackson ImmunoResearch Labs (West Grove, PA). Cationized ferritin-tetra methylrhodamine (TMR) and ferritin-TMR were purchased from Molecular Probes. Cholera toxin B subunit, α-amylase, human lactoferrin, chicken lysozyme, human lysozyme, myoglobin, and pepsin were all purchased from Sigma (St. Louis, MO). Proteins that were not already fluorescently labeled were labeled with FITC according to the manufacturer's instructions. Fluorescently labeled microspheres were purchased from Polysciences, Inc (Warrington, PA).

Human papilloma virus-like particles (VLPs) were a gift from Dr. John Schiller at the National Cancer Institute and Dr. Richard Roden at the Johns Hopkins School of Medicine. Norwalk VLPs were a gift from Dr. Mary Estes at the Baylor College of Medicine. VLPs were labeled with any of these four fluorophores: FITC, 5-iodoacetamidofluorescein, tetramethylrhodamine-5-(and-6)-isothiocyanate (Molecular Probes) and iodoacetamido tetramethylrhodamine (Research Organics, Inc, Cleveland, OH). VLPs were labeled in 10 mM phosphate-buffered saline (PBS, Sigma) with 0.02% sodium azide at 50 μg label/mg of VLP. The reaction was allowed to proceed at 4°C for 3 h before being quenched with excess glycine (Sigma). The mixture was sonicated (bath sonicator, Branson B-220, VWR, West Chester, PA) five times at 4°C for 15 s with a 15-s rest between each pulse. Free dye was removed by chromatography on either Sepharose CL-2B (0.7 $\times$ 48 cm, Amershan Pharmacia Biotech, Piscataway, NJ) or 10DG (10 ml desalting column, Bio-Rad, Hercules, CA).

Herpes simplex virus 1 (HSV) with green fluorescent protein attached to the capsid protein L2 (Desai and Person, 1998) was a gift from Dr. Prashant Desai at the Johns Hopkins School of Medicine.

Diffusion measurements

Two methods were used to measure diffusion of probes in mucus. FRAP has been described previously (Axelrod et al., 1976; Wolf, 1989). An attenuated laser beam is focused through a modified epifluorescent microscope to a small point (~4 μm in diameter) in a sample (mucus, saline, water, etc.) containing the fluorescent probe of interest (HPV, antibodies, etc.). The attenuator is transiently removed to increase the intensity of the beam approximately 10^3- to 10^4-fold, which rapidly and irreversibly bleaches a fraction (typically 20–50%) of the fluorescent molecules in this part of the sample. After a bleach exposure lasting 10–40 μs, the attenuator is replaced in the laser beam and the residual fluorescence from the same area in the sample is monitored. As unbleached probes diffuse into the partially bleached area, the fluorescent signal returns to a new steady-state value. The rate at which the fluorescence recovers reveals the rate of diffusion of the fluorescent particles. Also, the fractional recovery of the fluorescent signal corresponds to the fraction of the fluorescent probes that are mobile.

In mucus samples with fluorescently labeled HSV, the fluorescent signal was not sufficiently uniform or sufficiently bright to perform useful FRAP experiments. In these cases, diffusion was observed with multiple image photography (MIP) of individual fluorescent particles. Using an epifluorescent microscope (600×, Nikon E-800, Tokyo, Japan), images of individual fluorescently labeled HSV in samples of mucus and saline were recorded with a charge-coupled device camera (Princeton Instruments Inc., Trenton, NJ). Two images of the same field were taken 1.5–50 s apart. IPLab Spectrum (Signal Analytics Corp., Fairfax, VA) was used to analyze the images. Successive images were subtracted from one another to reveal the particle displacements, $\triangle$r, that occurred between images. The diffusion coefficient was calculated using the equation: $D = \triangle r^2/4\triangle t$. This system was validated by measuring diffusion coefficients for fluorescent latex microspheres of well defined diameter in PBS.

Samples were prepared similarly for both FRAP and MIP. Aliquots of proteins or VLPs were sonicated for 15 s three times to minimize aggregation (20% power, duty cycle 2, 15 s rest between each sonication, Branson Sonifier, VWR). Aliquots of cervical mucus (0.01–0.04 g) were measured on a balance. A 1:10 (v/w) dilution of 20 mg/ml bovine serum albumin (Sigma) was added to samples that did not already contain bovine serum albumin and stirred to help prevent fluorescently labeled probes from sticking to the glass. Finally, the fluorescent probe was added (1:10 v/w dilution) and stirred by hand with a glass rod at least 50 times. This method diluted the mucus no more than 20%, and because the average mesh spacing between mucin fibers varies inversely with the square root of mucin concentration, this 20% dilution probably increased the average mesh spacing by $\leq$10%. For FRAP samples, the mucus was then drawn by suction into a flat capillary tube (microslides, 0.3 × 3.0 mm ID, VitroCom Inc., Mt. Lakes, NJ) which was sealed with Critoseal (VWR). For MIP samples, the mucus was placed on a microscope slide to which a small piece of double-sided tape (3M, St. Paul, MN) that had a ~1 cm × ~1 cm square cutout was applied. This sample area was then sealed with a coverslip. The double-sided tape ensured that the sample was not compressed excessively. The capillary tubes, microscope slides, and coverslips were all pretreated with silicon (Rain-X, Unelko Corp., Scottsdale, AZ) to minimize sticking of the labeled probes to the glass.

EM

Fresh HCM samples were drawn into sterile catheters (Infant Feeding Tube, 8 FR × 15″, Mallinckrodt Baker, Glens Falls, NY) and the catheter was then cut into 5-mm long sections. These mucus-containing sections were immersed for 1 h in fixative (10% dimethylsulfoxide (DMSO), Mallinckrodt Baker), 2% glutaraldehyde in 0.1 M cacodylate (Electron Microscopy Sciences (EMS), Fort Washington, PA)). The mucus samples were then placed into 100% DMSO and plunged into liquid propane that had been cooled to −160°C with liquid nitrogen. After 30–60 s, the samples were removed from the liquid propane and placed in 100% methanol (Mallinckrodt Baker) precooled to −80°C. Samples were left in 100% methanol at −80°C for 4–6 days, then placed in 1% OsO_4 (EMS) in 100% methanol (precooled to −80°C). Samples were left at −80°C for 2–4 h, moved to −20°C for 8 h, and then left at room temp for 1 h. Finally, samples were washed with 100% methanol, removed from the catheters, and embedded in epon (EMbed 812, EMS). Sections were cut with a diamond knife (Delaware Diamond Knives, Wilmington, DE) and placed on grids (EMS) for observation with a Phillips 410 electron microscope at 80 kV (Phillips, Amsterdam, Netherlands).

RESULTS

The results of experiments on the diffusion of macromolecules and viruses in human midcycle cervical mucus are compiled in Table 1. The ratio of diffusion coefficients, D_{muc}/D_{pbs}, indicates the speed with which a probe diffuses in mucus compared with saline buffer. If this ratio = 1, then the probe diffuses as rapidly in mucus as in buffer, whereas a value <1 implies that the probe is slowed by mucus.

Most proteins (15–650 KDa) diffused as fast in mucus as they did in water (D_{muc}/D_{pbs} = 0.84–1.1) and had > 93% recovery. Three proteins, human lysozyme, cholera toxin B subunit, and cationized horse ferritin seemed to be slowed slightly (D_{muc}/D_{pbs} = 0.68–0.75), but the error limits indicate that these observations were not significantly <1. However, three Ig molecules were slowed significantly (D_{muc}/D_{pbs} = 0.24–0.51). These were IgM, IgM $Fc_{5\mu}$ (the Fc pentamer core of an IgM molecule with all 10 Fabs removed), and small aggregates of sIgA molecules. Based on hydrodynamic radius inferred from D_{pbs}, the sIgA samples are estimated to be aggregates of 6 to 10 Ig molecules. Not only was the diffusion of these Igs slowed, their percentage recovery was somewhat lower.

Surprisingly, two VLPs diffused as fast in mucus as they did in saline. These were Norwalk VLP (38-nm diameter) and HPV VLP (55-nm diameter). Not only did the VLPs readily diffuse in mucus, their percentage recovery was 85% and 76%. The diffusion coefficients measured in saline for both VLPs matched what would be expected for particles their size, ensuring that VLPs and not capsid monomers were being observed. Further, when the samples were observed with epifluorescence at 600× magnification, the fluorescence was uniform throughout the mucus sample, indicating that Norwalk and HPV did not adhere to mucus strands nor were they excluded from areas of concentrated mucins.

Unlike the diffuse fluorescence of HPV samples, fluorescently labeled polystyrene microspheres (59 nm to 1 μm) mixed into mucus stuck tightly to the mucins and collapsed the mucus gel into thick "cables" of aggregated mucin strands (Fig. 1). FRAP experiments showed no recovery after bleaching, thus $D_{muc} = 0$ for microspheres, regardless whether the microspheres were unmodified polystyrene or if their surfaces were functionalized with carboxylate, epoxy, or amino groups.

Similarly, visual observation of HSV in mucus showed that HSV colocalized with mucus strands in the sample (Fig. 2). Also, HSV in mucus did not visibly move during direct visual observations, unlike HSV in PBS, which could be observed moving within the expected range of thermal velocities. In some mucus samples, there were regions where the mucus appeared thinner and HSV was more evenly distributed. In these regions, MIP was performed. At least eight different mucus samples were observed and displacements for approximately 100 virions were obtained per sample. Comparing these observations with diffusion of HSV in PBS, the HSV in regions of thin mucus was slowed 100- to 1000-fold ($D_{muc}/D_{pbs} = (8.9 \pm 5.2) \times 10^{-3}$). To ensure that the sticking or cabling of HSV in mucus was not caused by interactions with anti-HSV antibodies, each of

TABLE 1 Diffusion of proteins and viruses in human cervical mucus

Probe	MW (kDa)	d (nm)	D_{muc}/D_{pbs}	% R	N	n
h lysozyme	15	3.5*	0.75 ± 0.23	96 ± 1	2	20
Horse myoglobin	17	3.8†	0.84 ± 0.21	96 ± 2	5	55
Chicken lysozyme	14	4.1†	1.1 ± 0.2	96 ± 4	2	21
Porcine pepsin	35	4.5†	0.98 ± 0.02	96 ± 1	4	32
h IgM Fab_{μ}	50	6.0‡	0.94 ± 0.23	94 ± 13	6	110
Cholera toxin B subunit	55	5.4*	0.68 ± 0.15	102 ± 2	5	60
h α-amylase	59	5.5*	0.90 ± 0.02	100 ± 2	2	28
h lactoferrin	82	6.1*	0.86 ± 0.21	101 ± 6	3	28
h IgG**	152	11†	0.87 ± 0.19	97 ± 1	5	51
h IgG**	152	11†	1.1 ± 0.1	98 ± 1	5	100
h IgA**	160	11§	0.85 ± 0.02	98 ± 1	2	30
h IgA**	160	11§	1.1 ± 0.2	97 ± 1	4	80
h IgM $Fc_{5\mu}$	~300	11§	0.41 ± 0.12	87 ± 4	6	120
Horse ferritin	650	12*	0.88 ± 0.18	93 ± 11	4	44
Cationized horse ferritin	650	12*	0.73 ± 0.32	97 ± 4	3	34
h IgM**	950	16§	0.51 ± 0.17	95 ± 4	5	56
h IgM**	950	16§	0.33 ± 0.11	82 ± 22	6	120
h sIgA aggregates		18§	0.24 ± 0.16	76 ± 11	4	33
Norwalk VLP	10,000	38¶	1.1 ± 0.4	85 ± 8	11	130
HPV VLP**	20,000	55‖	1.1 ± 0.2	76 ± 17	18	209
HPV VLP**	20,000	55‖	1.3 ± 0.4	92 ± 10	3	50
HSV††		~180	0.0089 ± .0052	n/a	8	768

h represents human proteins. d is the Stokes diameter (nm) for the probes.

*Calculated from the Stokes-Einstein equation ($d = kT/3\pi\eta D_{water}$ where k is Boltzmann's constant, T is absolute temperature, η is the viscosity of water, using D_{water} taken from Sober, 1970.

†Calculated from the Stokes-Einstein equation where diffusion coefficients were unknown and were calculated from $D_{water} = A(M_w)^{-1/3}$ where M_w is given by the manufacturer and $A = 300\ cm^2(Da)^{1/3}s^{-1}$ (Polson, 1950).

‡Calculated from the Stokes-Einstein using D_{water} (Saltzman et al., 1994).

§Calculated using Stokes-Einstein with D_{pbs} using IgG as a standard.

¶EM of Norwalk VLPs (Venkataram Prasad et al., 2000).

‖EM of HPV VLPs (McCarthy et al., 1998). %R is the percentage fluorescent recovery. N is the number of mucus samples observed. n is the total number of recovery curves observed for FRAP experiments and total number of virions tracked for MIP experiments.

**probes listed twice were tested by two investigators at different times and are reported separately.

††HSV results are from MIP experiments, all other probes are from FRAP experiments.

these mucus sample was tested for the presence of anti-HSV antibodies by an immunofluorescent assay (Gallo, 1986). None of the mucus samples used for HSV experiments contained detectable anti-HSV antibodies by immunofluorescent assay.

The mesh spacing in a typical sample of HCM fixed with glutaraldehyde delivered in DMSO is revealed by EM (Fig. 3). Throughout the gel, the mesh spacing ranged from 20–200 nm and the individual fibers had a diameter of ~5–15 nm.

DISCUSSION

Two major mechanisms may stop particles from readily diffusing through a mucus gel. Particles can stick to mucin fibers or they can be hindered by the size of the mesh spacing between the mucin fibers. We studied the diffusion of fluorescently labeled proteins, VLPs, an enveloped virus, and polystyrene microspheres in fresh, only slightly diluted samples of human midcycle cervical mucus. Most of the proteins tested were able to diffuse in these samples of mucus as fast as they diffuse in water, indicating that the size of the mesh-spacings in these mucus samples did not significantly hinder or block the diffusion of any proteins tested. These results confirm earlier studies that showed small molecules and proteins could diffuse in mucus as fast as they diffused in water (Radomsky et al., 1990; Saltzman et al., 1994) and expand the number of different proteins that have been studied.

Most surprising were the results that Norwalk and HPV VLPs, two large multimeric protein capsid assemblies, diffused in mucus as fast as they diffused in saline buffer. VLPs are virus protein capsids that contain no nucleic acid. Their external surfaces are the same as infectious virions, but they are safer to handle and use experimentally and can be produced in large quantities. Norwalk VLPs are 38 nm (Venkataram Prasad et al., 2000) and HPV VLPs are 55 nm (Rose et al., 1993) in diameter with molecular masses of ~10,000 kDa and ~20,000 kDa, respectively. Thus, these VLPs are much larger than any particles previously shown to diffuse unhindered in mucus. In contrast, a larger enveloped virus, HSV (180 nm), did *not* readily diffuse in mucus and also seemed to stick to and colocalize with the mucin strands.

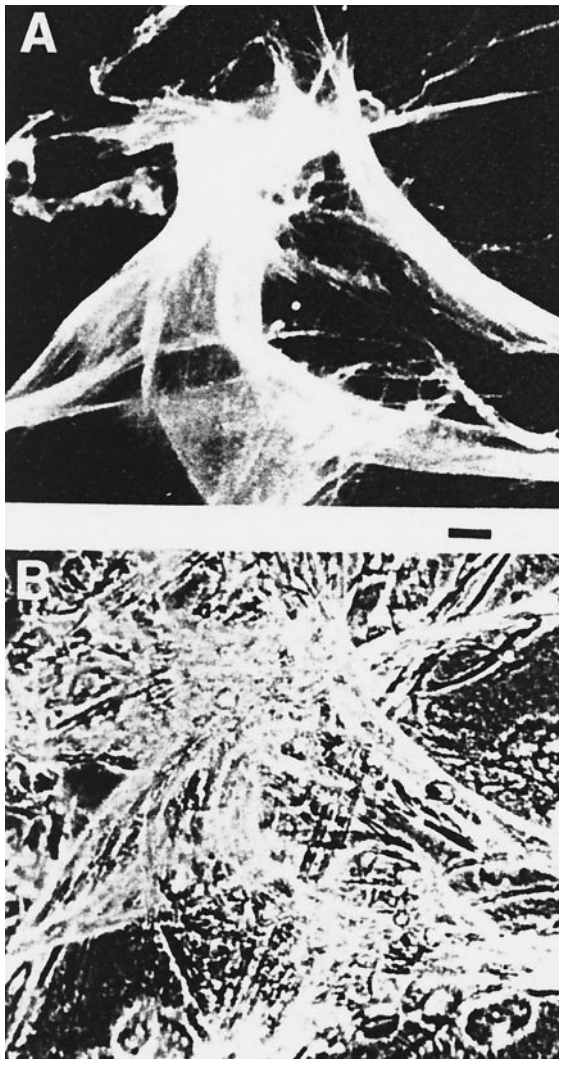

FIGURE 1 Polystyrene microspheres in HCM. Fluorescently labeled 59 nm microspheres formed cables with mucins in the mucus gel. (*A*) Fluorescent image. (*B*) Phase image. Scale bar = 500 μm.

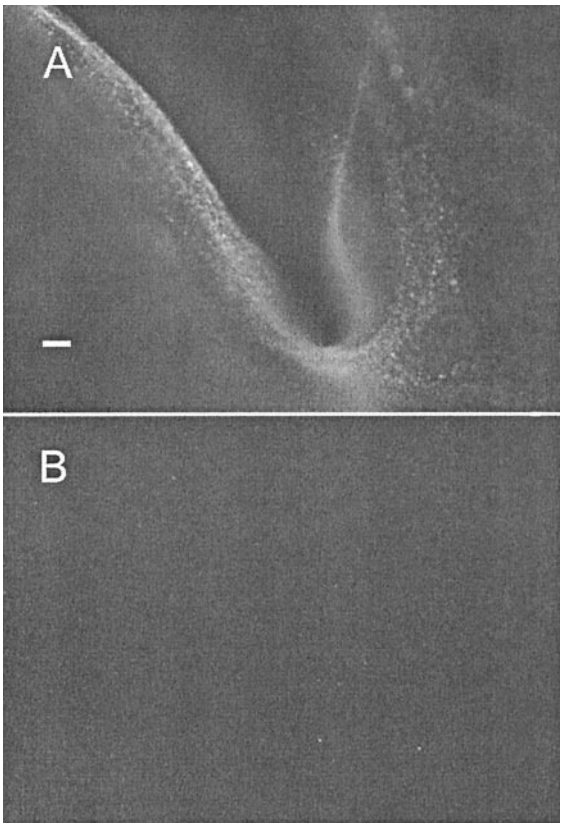

FIGURE 2 Herpes simplex virus in HCM and PBS. Fluorescent images of HSV are shown in mucus (*A*) and PBS (*B*). Note that HSV seems to colocalize with strands of mucus, but was randomly dispersed in PBS. Scale bar = 20 μm.

Polystyrene microspheres 59–1000 nm in diameter, similar in size to the virus particles, seemed to adhere even more tightly to mucin fibers than to HSV, and formed dense cables (Fig. 1). Even with the smallest microsphere, 59 nm, no diffusion of microspheres was detectable with FRAP. These observations are consistent with the observations that microspheres orally ingested by the rat are trapped in the mucus gel of the gastrointestinal tract (Jani et al., 1992). In striking contrast, both HPV and Norwalk VLPs diffused unhindered through mucus. Because these viruses are constructed with a large number of capsid monomers that could potentially make highly polyvalent bonds between the virus particle and the mucus gel, to diffuse through mucus without slowing, Norwalk virus and HPV must have evolved external capsid surfaces that do not make any bond, even very low-affinity bonds, with mucins. Capsid monomers might minimize affinity bonds to mucins by being densely coated with both positive and negative charges, an adhesion-preventing mechanism present on the surface of most soluble proteins (Wada and Nakamura, 1981). In addition, capsid viruses must have few, if any, exposed hydrophobic regions that can make highly polyvalent bonds with the hydrophobic domains distributed along mucin fibers (Cone, 1999).

Nonmotile mucosally transmitted pathogens must usually diffuse to contact their target cells. These experiments suggest that small capsid viruses are capable of diffusing through the protective mucus coat to reach these target cells. In fact, many protein capsid viruses are smaller than 55 nm, with polio virus being ~30 nm and rotavirus being the largest at ~75 nm (Fields et al., 1996). In contrast, HSV, an enveloped virus, sticks to mucus and will therefore be trapped by the mucus gel. Thus, HSV seems most likely to infect cells not coated with mucus, such as cells exposed by abrasion.

Most soluble proteins tested were not significantly slowed in mucus. However, IgM, IgM-Fc$_{5\mu}$, and small aggregates of sIgA were slowed 3- to 5-fold in mucus. These Igs are significantly smaller than the Norwalk and HPV VLPs that were not slowed in mucus. Thus, these Igs

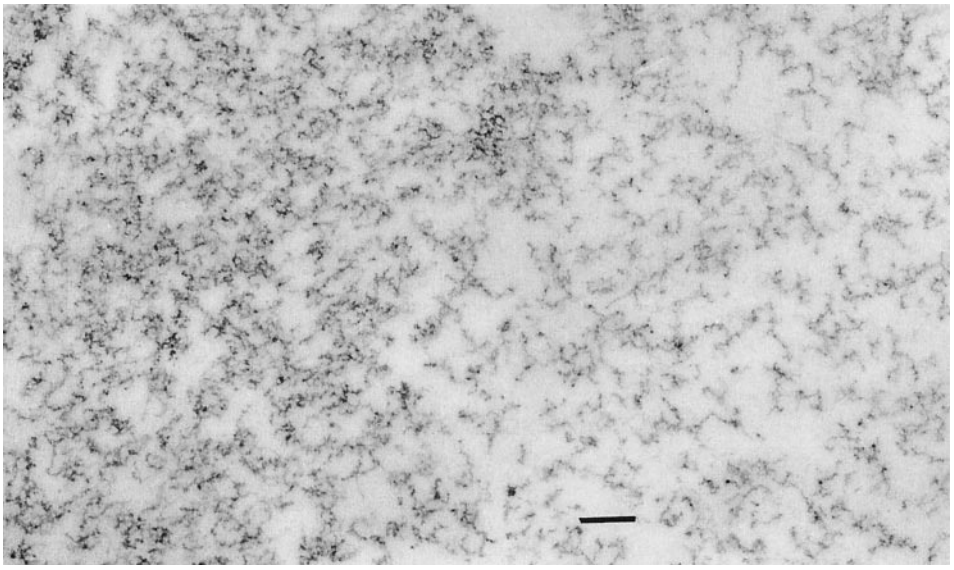

FIGURE 3 Transmission EM of HCM. This is a typical image of fixed HCM (~80 nm thick). Note the interfiber spacing varies between regions of low and high density, but ranges from 10 s to 200 nm. Similarly, individual fibers can be observed with fiber diameters ~10 nm. Scale bar = 200 nm.

must not be hindered by the mesh spacing in mucus. Instead, Igs seem to be slowed by making low-affinity bonds with the mucins. That antibodies must bind to mucins has been inferred from the shaking phenomenon, in which antisperm antibodies (IgG, IgA, or IgM) that accumulate on the sperm surface trap individual vigorously motile sperm in mucus; such antibody-coated sperm shake in place for hours and never swim free unless the mucus gel is highly diluted (Jager et al., 1981; Kremer and Jager, 1976). The binding between antibodies and mucins must have very low affinity because the diffusion of IgG, with only one Fc region, was not slowed significantly in mucus but the antibodies with multiple Fcs were significantly slowed. Unlike IgG, IgM, with 5 Fcs, and small aggregates of sIgA with 6 to 10 Fcs are capable of forming multiple low-affinity bonds with mucins. In fact, although there are many experimental results that indirectly indicate that mucins bind antibodies with low affinity (Biesbrock et al., 1991; Kremer and Jager, 1976; McSweegan et al., 1987; Miller et al., 1981), several biochemical methods have failed to detect or characterize the low-affinity bonds that must form between antibodies and mucus (Crowther et al., 1985; Iontcheva et al., 1997). The results reported here indicate that it is the Fc moiety of an antibody that binds to mucins, consistent with shaking phenomenon experiments (Jager et al., 1981). Cleaving the Fab molecules from an IgM leaves a pentameric ring of Fcs joined by the IgM J-chain. The diffusion of this pentameric Fc ring (IgM $Fc_{5\mu}$) was slowed to the same extent as the whole IgM. If the Fab portion of antibodies made significant affinity bonds with mucins, then removing all 10 Fabs from an IgM would be expected to markedly increase the speed of diffusion of the IgM $Fc_{5\mu}$. Because no change was detected, it must be the pentameric Fc ring that makes low-affinity bonds with mucins.

We have used a novel approach to prepare mucus for EM. During fixation, DMSO serves as a carrier, which elevates the penetrability of the glutaraldehyde and thereby allows rapid diffusion of the fixative well into the gel, minimizing artifacts of cabling and outer-surface condensation. Unlike previous EM preparations, these images, showing fiber diameters of 5–15 nm and mesh spacing of 20–200 nm, are consistent with biochemical, AFM, and EM studies of individual mucin monomers (McMaster et al., 1999; Slayter et al., 1991; Yurewicz et al., 1987).

Many empirical models have been developed to analyze diffusion in polymer gels (Amsden, 1998). The model that best fits experimental data is the obstruction-scaling model that was developed for covalently cross-linked hydrogels but is equally applicable to gels with physical entanglement cross-links (Amsden, 1998, 1999). This model treats cases in which the effective radius of the mesh spacing is greater than the hydrodynamic radius of the diffusing particle and assumes no interaction between the solute and the polymer (Amsden, 1999). The model describes the ratio of diffusion in a gel and diffusion in water as:

$$D_g/D_o = \exp\{(-\pi/4)[(r_s + r_f)/(r_g + r_f)]^2\}$$

where D_g is the diffusion coefficient of the particle in the polymer gel, D_o is the diffusion coefficient of the particle in water, r_s is the radius of the particle, r_f is the gel fiber radius, and r_g is the effective radius of the mesh spacing.

This model was developed for chemically cross-linked hydrogels such as poly(ethylene oxide), poly(vinyl alcohol), and poly(acrylamide), among others. These hydrogels have a very small fiber radii (~0.2–0.3 nm) and well characterized repeating chemical subunits. In contrast, mucin fibers are 10–50 times thicker, and have markedly more chemical variety because of their complex sugar branches interspersed between stretches of relative hydrophobic peptide sequences. However, mucins are long, linear, polymeric chains which, although not chemically cross-linked, do

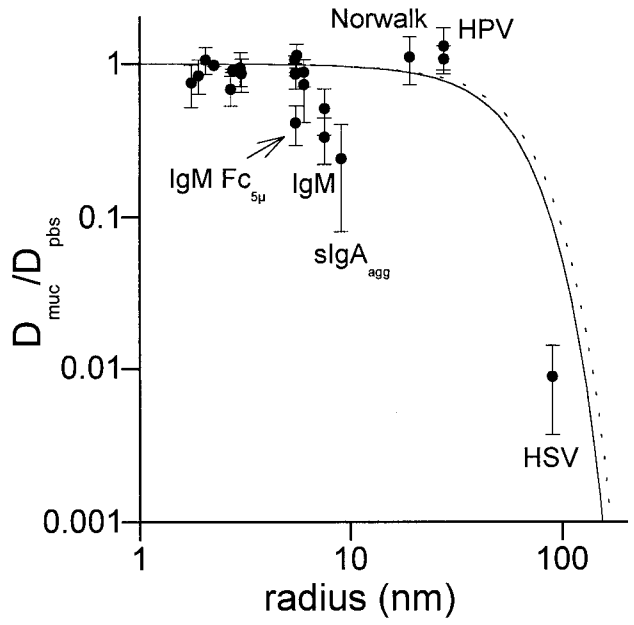

FIGURE 4 Normalized diffusion coefficients for proteins and viruses in mucus. $D_{muc}/D_{pbs} \pm$ SD is plotted for the particles listed in Table 1. If a particle diffuses in mucus as fast as it diffuses in saline, $D_{muc}/D_{pbs} = 1$. The lines drawn on the graph are the ratio predicted by Amsden's obstruction-scaling model. The *solid line* uses a mucin fiber radius of 3.5 nm and mesh fiber spacing of 100 nm. The *dotted line* takes into account the 20% dilution of the mucus samples in our experiments by increasing the mesh fiber spacing by 10%.

form a physically entangled gel that may be further stabilized by low-affinity bonds between hydrophobic domains (Cone, 1999).

We have applied Amsden's obstruction-scaling model to HCM and have plotted the diffusion ratios predicted by this model in Fig. 4. We used a mucin fiber radius (r_f) of 3.5 nm which is our best estimate from biochemical, EM, and AFM observations of individual mucin fibers and our images of mucus gels. We calculated the mesh spacing within a mucus gel using a simple cubic-lattice model of cylindrical fibers. This cubic-lattice model predicts a mesh spacing of 100 nm, which is consistent with the values we observe in EM of fresh HCM (Fig. 3). Because the mucus samples were diluted 20% in the process of adding the fluorescent probes, the radius of the average mesh fiber spacing would increase by ~10%. We plotted Amsden's model with both 50 nm (*solid line*) and 55 nm (*dotted line*) r_g to show the limited effect that a 10% change in mesh fiber spacing has on diffusion in the mucus gel. Therefore, we would not expect diffusion of proteins and viruses in vivo to differ significantly from what we observe in these slightly diluted samples.

As indicated in Fig. 4, Amsden's model predicts that HPV and Norwalk would diffuse essentially unhindered in mucus. Also, the model predicts D_{muc}/D_{pbs} for HSV would be reduced by ~10-fold. However, because HSV colocalized with mucin strands and only diffused in regions of mucus that seemed to be thinner, our observations suggest that HSV is slowed not only by the mesh spacing but also by making multiple low-affinity bonds to mucin fibers. Whereas Amsden's model indicates a sharp cutoff for particles diffusing in a gel, only HSV fell within the sharp cutoff range. Therefore, although our results with HPV provide a lower limit on the mesh spacing of human midcycle cervical mucus, and because HSV binds lightly to mucin fibers, our results do not provide an upper limit on mesh spacing.

Mucus gels have long been considered a diffusional barrier for the surfaces they cover. Our data suggest, however, that most proteins and even capsid viruses can diffuse through mucus unhindered. In fact, mucus only trapped a larger enveloped virus, HSV. However, because Igs seem to make low-affinity bonds with mucins, and the rate of diffusion decreases with the number of Fc moieties per diffusing particle, our results suggest that when antibodies accumulate on the surface of a pathogen, they may be able to form a sufficient number of low-affinity bonds to trap pathogens in the gel, similar to what is seen for sperm (Kremer and Jager, 1976; Cone, 1999) as well as bacteria (Biesbrock et al., 1991; McSweegan et al., 1987).

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