Antibody Diffusion in Human Cervical Mucus

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ABSTRACT The mucosal immune system actively transports large quantities of antibodies into all mucus secretions, and these secreted antibodies help prevent infectious entry of many pathogens. Mucus is generally thought to protect epithelial cells by forming a diffusional barrier through which only small molecules can pass. However, electron microscopy indicates that the pore size in mucus is ~100 nm, which suggests that antibodies as well as other large molecules might also diffuse through mucus. We measured the diffusion coefficients for antibodies and other proteins within human midcycle cervical mucus using two techniques: fluorescence imaging of concentration profiles and fluorescence photobleaching recovery. The two techniques are complementary, since the rates of diffusion are observed over millimeter distances with fluorescence imaging of concentration profiles and micron distances with fluorescence photobleaching recovery. Both methods yielded essentially the same diffusion coefficients. In contrast to previous reports indicating mucus significantly impedes diffusion of small molecules, antibody diffusion in mucus was relatively unimpeded. In our observations IgG, IgG fragments, IgA, and IgM diffused almost as rapidly in cervical mucus as in water ($1.0 > D_{mucus}/D_{water} > 0.7$). Simple models for diffusion through water-filled pores suggest that the hydrodynamic pore size for cervical mucus is ~100 nm, smaller than the ~1000 nm pore size of a collagen gel (at 1 mg/ml) and larger than the ~10 nm pore size of gelatin (at 100 mg/ml). This estimated pore size is consistent both with electron micrographs and geometric models of interfiber spacing. Based on these results, we predict that particles as large as viruses can diffuse rapidly through human midcycle cervical mucus, provided the particle forms no adhesive interactions with mucus glycoproteins.

INTRODUCTION

The mucus secretions of the intestinal, respiratory, and reproductive tracts provide essential protection against the infectious entry of pathogens. Mucus is a hydrated gel composed of specialized glycoproteins (mucins) that are secreted by specialized epithelial cells (goblet cells). By continuously secreting and shedding a viscoelastic coating over the epithelial cells, a nonspecific mechanical barrier to pathogen entry is created. The mucin gel also contains secreted antibodies, which enhance protection against specific pathogens by trapping or immobilizing the pathogens in the mucus and inhibiting the ability of pathogens to adhere to target cells (Biesbrock et al., 1991; Magnusson and Stjernstrom, 1982; McSweegan et al., 1987; Tse and Chadee, 1991).

It appears likely that secreted antibodies, if they are to provide effective immune surveillance within a mucus secretion, must be able to diffuse through the mucin gel, since the speed with which antibodies can accumulate on the surface of a pathogen in mucus is likely to depend on the rate of antibody diffusion through the mucus. Unfortunately, only a few measurements of antibody diffusion rates in animal tissues have been reported (Clauss and Jain, 1990) and, in spite of its importance to immune protection, rates of antibody diffusion in the secretions have never been measured. In general, however, it has been assumed that macromolecules, like proteins, diffuse slowly if it all within the mucus secretions (see Lamont, 1992, for a recent review of the barrier properties of mucus). That mucus slows the diffusion of macromolecules has been inferred using indirect techniques or by measuring the rates of permeation through a thin layer of mucus sandwiched between filters facing stirred reservoirs containing unequal concentrations of solute (Desai et al., 1992; Desai and Vadgama, 1991).

Unfortunately, although this method is conceptually straightforward, it is also sensitive to parameters that are difficult to control, such as the thickness of the unstirred layer outside the filters, the thickness of the mucus sample, blockage of filter pores by mucus, and alterations in mucus properties that occur either during preparation or when the mucus is exposed to the fluids in the reservoir. In the few cases in which the diffusion of macromolecules was examined with these methods (Desai et al., 1992; Murty et al., 1985; Smithson et al., 1981), the estimated diffusion rates through mucus were low, but the mucus never completely blocked diffusion of the macromolecules tested.

Although the previous literature implies that mucus greatly slows diffusion of macromolecules, electron microscopy reveals interfiber spacings in mucus that are much larger than macromolecules. Most globular macromolecules are less than 10 nm in diameter, but the most detailed studies of human cervical mucus structure, which employed freeze-substitution fixation and transmission electron microscopy (Yudin et al., 1989), reveal that mucus has a fibrous structure with interfiber spacings of ~500 nm between the primary elements and an additional finer structure with ~100 nm spacing. These observations of the interfiber spacings in mucus are not consistent with the general impression that mucus presents a significant diffusion barrier to macromolecules. In the present report, we attempted to resolve this inconsistency

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by measuring rates of diffusion for 11 proteins—from small globular proteins to the largest antibodies—within fresh, minimally perturbed samples of human cervical mucus.

Our studies were also motivated by the need to develop methods for delivering antibodies to immunize mucosal surfaces. Specific antibodies delivered to mucus secretions have been shown to protect against intestinal pathogens (Tacket et al., 1988) and sexually transmitted disease pathogens (Zeitlin et al., 1993), and antisperm antibodies added to semen can block fertilization (Menge and Protzman, 1967; Naz et al., 1983; O'Rand, 1981). Polymers that control the release of antibodies may provide a useful technique for the sustained delivery to mucus secretions. In previous reports, we demonstrated that biologically active antibodies can be released from devices made with biocompatible polymers (Saltzman et al., 1993; Sherwood et al., 1992). These polymer devices provided continuous delivery of antibodies to the mucus secretions of the mouse vagina for 30 days (Radomsky et al., 1992). Antibodies delivered to mucosal surfaces by a polymer device must be able to diffuse readily through the mucus if they are to become well distributed over the mucosal surface. Therefore, we evaluated the ability of antibodies released from a polymer device to diffuse through unstirred human cervical mucus.

MATERIALS AND METHODS

Materials

Poly(ethylene-co-vinyl acetate) (EVAc: Elvax 40W, DuPont, Wilmington, DE) was washed for 48 h in water and 48 h in acetone in a Soxhlet extractor to remove impurities. Fluorescein (Sigma Chemical Co., St. Louis, MO) was used as received. Fluorescein isothiocyanate- conjugated bovine serum albumin (FITC-BSA, Sigma) was mixed 1:24 with unlabeled bovine serum albumin (BSA, Sigma), reconstituted in distilled water, and lyophilized to create solid particles. A small chemotactic polypeptide (formyl-Nle-Leu-Phe-Tyr-Lys fluorescein derivative, Molecular Probes, Eugene, OR) was mixed 1:53 with glucose (Sigma), reconstituted, and lyophilized. FITC-conjugated ovalbumin (Sigma), lactalbumin (Sigma), and IgG (human serum, Jackson ImmunoResearch Laboratories, West Grove, PA) were mixed 1:19, 1:14, and 1:30 with Ficoll (mol wt 70,000, Sigma), reconstituted, and lyophilized. Fluorescein labeled-secretory IgA (S-IgA, human serum, Jackson) and IgM (human myeloma, Jackson) were mixed 1:26 and 1:52, respectively, with Ficoll (mol wt 400,000, Sigma) and lyophilized. The lyophilized FITC conjugates were crushed to obtain 50- to 500-μm solid particles.

Fabrication of EVAc polymer matrices

Polymer matrices were fabricated by solvent evaporation (Radomsky et al., 1990; Rhine et al., 1980; Saltzman and Langer, 1989). Solid particles containing fluorescent molecules were added to a 10% (w/v) solution of EVAc in methylene chloride. Sufficient solid particles were added to the polymer solution so that the mass fraction of particles in the resulting polymer/particle matrix was 35%. The solution was vortexed and poured into a chilled glass mold (2 × 2 cm; −80°C). After solidification (about 10 min), the slab was maintained at −20°C for 48 h and 25°C under vacuum (10–50 mm Hg) for an additional 48 h. During this period, the methylene chloride was evaporated from the slab.

Characterization of mucus samples

Midcycle human cervical mucus, in which sperm can maintain good forward motility, was obtained from women at The Johns Hopkins Infertility Clinic and Union Memorial Hospital (Baltimore, MD). Samples were stored at 4°C until use, generally within one week of collection. Only samples that were optically clear, and therefore relatively free of cellular debris, and that exhibited spinnbarkheit (i.e., capacity to be drawn into threads) of more than 3 cm were used. The pH of cervical mucus samples was determined with pH paper; a pH range of 8.0–9.0 was found for 12 representative samples (mean pH 8.6).

The total dry weight of molecules with molecular weight of more than 6000 was determined by gravimetric analysis. A small amount (50–200 mg) of mucus was added to a short length (~5 cm) of molecular porous membrane tubing (Spectra/por, 10 mm wide, 6000–8000 mol wt cutoff, 6.4 mm dry cylinder diameter). The sample was dried overnight in a vacuum desiccator. The total weight of dry solids was then determined. The ends of the tube were closed and the sample was placed in agitated distilled water (1 l). The water was replaced after 6 h and again after 12 h. After 24 h, the sample was again dried in a vacuum desiccator and the total dry weight greater than 6000 kDa was determined.

Midcycle human cervical mucus samples were fixed in 2% glutaraldehyde in PBS for 1 h. After two washes with PBS, they were post-fixed in 1% osmium tetroxide (OsO_4) in PBS for 0.5 h. After three washes in PBS, they were dehydrated through a series of ethanol solutions, critical-point dried with liquid CO_2, and sputter-coated with a gold-platinum alloy. The resulting samples were examined by scanning electron microscopy.

Formation of collagen and gelatin gels

Solutions of type I collagen were prepared from rat tail tendons as previously described (Bell et al., 1979). Briefly, tendons from the tails of ~300-g Fisher 344 rats were extracted, washed with 70% ethanol, and dissolved in 0.02 M acetic acid (2 days at 4°C). The resulting solution was centrifuged (15,000 rpm for 45 min) and the collagen supernatant solution was retained and stored at 4°C. As previously described (Parkhurst and Saltzman, 1992; Saltzman et al., 1992), the purity of the collagen solution was determined by SDS-PAGE and the concentration of the collagen solution was determined by gravimetric analysis and a bicinchoninic acid protein assay. To form a gel, the osmolarity and pH of the collagen solution was raised to physiological levels (300 mOsm, pH 7.4) by adding 0.1 M NaOH, 5% sodium bicarbonate, and highly purified distilled water. Before gelation, a small amount of solution (25–100 μl) was drawn into a flat glass capillary tube and incubated at 37°C. The gel completely set within 10 min. Gelatin samples (100 mg/ml) were prepared by adding gelatin (Knox) to distilled water and heating briefly to dissolve. A small amount (25–100 μl) of hot gelatin was drawn into a flat glass capillary tube and allowed to cool.

Measurement of diffusion coefficient by fluorescence imaging of profiles

The diffusion coefficient of molecules released from a polymer matrix into an adjacent unstirred fluid was determined by computer imaging of fluorescence profiles (FIP) near the edge of a polymer test slab (Radomsky et al., 1990). A polymer matrix was cut into test slabs (3 × 1 × 0.4 mm) and each test slab was inserted in the open end of a flat glass capillary tube (50 × 5 × 0.5 mm, Vitro Dynamics, Rockaway, NJ) previously filled with phosphate- buffered water (pH 7.4), midcycle human cervical mucus, collagen gel (1.0 mg/ml), or gelatin gel (100 mg/ml). To minimize convection currents, the capillary tube was mounted vertically with the test slab on the bottom. This insured that the highest density fluid was at the bottom of the tube. The tube was viewed through a horizontally mounted microscope objective in a modified epifluorescence inverted microscope (Nikon Diaphot, Garden City, NY). The microscope was equipped with a video camera (model NC-70, Dage-MTI, Wabash, MI) connected to a computer (Compaq 386/20e, Houston, TX) with frame grabber (Data Translation model 2851/2858, Marlborough, MA) permitting quantification of the light intensity at every point in the image. At various times after insertion of the polymer into the fluid-filled capillary tube, the tube was scanned with the fluorescence beam; the relative concentration at any position in the sample was obtained from the normalized fluorescence intensity. Once the concentration profiles

(concentration, C, as a function of position and time) were stored in the computer, the diffusion coefficient of the molecule of interest was calculated. Details regarding both calibration of the imaging system and analytical methods for calculating the diffusion coefficient in the unstirred fluid, as well as sample curves showing fits to the data, were reported previously (Radomsky et al., 1990).

Measurement of diffusion coefficients by fluorescence recovery after photobleaching (FPR)

A flat glass capillary tube was filled with phosphate- buffered water, human midcycle cervical mucus, or collagen gel. As above, a small test slab of polymer containing fluorescent probe was inserted into the filled tube. The tubes were incubated at room temperature overnight to permit the labeled molecules sufficient time to diffuse into the adjacent mucus and, importantly, to permit the mucus or gel to relax sufficiently to become essentially immobile during the course of each measurement. The computer-controlled instrument for photobleaching has been described previously (Wolf, 1989). An attenuated laser beam was focused near the center, i.e., between the walls, of the capillary tube. Label was bleached by briefly removing the attenuation, increasing the light intensity in the spot by 3–4 orders of magnitude for 5–30 ms. The attenuated beam was then used to measure the time course of recovery of fluorescence in the bleached spot. The fraction of labeled molecules that were mobile, M_f, and the diffusion coefficient of mobile molecules, D_i^{FPR}, were both determined from the recovery curve, as previously described (Barisas and Leuther, 1977). In all of the results reported here, the fraction of mobile molecules, M_f, was near unity.

RESULTS

Solid particles containing the fluorescence-labeled proteins were dispersed into matrices of EVAc. When an EVAc/protein matrix was placed in the bottom of a vertical capillary tube filled with buffered water, the protein diffused upward out of the polymer matrix into unstirred water. By measuring concentration profiles in the vicinity of the polymer using epifluorescence microscopy, and comparing these profiles to solutions to the diffusion equation, the diffusion coefficient for the fluorescent protein in water was determined (Fig. 1). The diffusion coefficients agree with previously published values obtained with a variety of other methods, giving us confidence in this new method, which we call FIP.

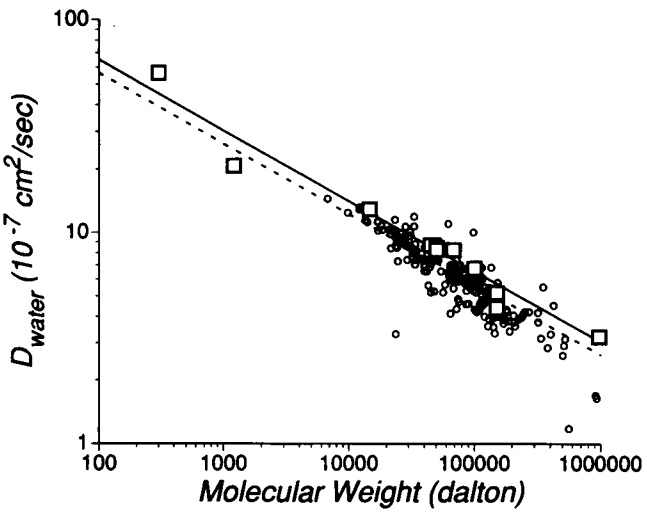

FIGURE 1 Diffusion coefficient in buffered saline is plotted as a function of molecular weight for fluorescein and fluorescence-labeled proteins (see Table 1 for a list of proteins and molecular weights). For each fluorescent molecule, the diffusion coefficient was measured by FIP (□). Each symbol represents the mean diffusion coefficient from three to five separate measurements; standard deviations were smaller than the symbols. For comparison, the smaller circles (○) indicate diffusion coefficients for globular proteins obtained from the literature (Sober, 1970). The *solid lines* indicate the best fit of Eq. 2 to the experimental (A = 260 cm^2/s-dalton$^{0.333}$) and literature values (A = 300 cm^2/s-dalton$^{0.333}$), consistent with the expected behavior of globular proteins.

For a spherical macromolecule diffusing through a medium consisting of much smaller solvent molecules, like water, the Stokes-Einstein equation can be used to correlate the diffusion coefficient, D_{water}, with molecular size (i.e., the hydraulic diameter, d) (Einstein, 1906):

$$D_{water} = \frac{kT}{6\pi\eta(d/2)} \quad (1)$$

where T is the absolute temperature, η is the solvent viscosity, and k is Boltzmann's constant. Since the diameter of

TABLE 1 Diffusion of fluorescein and fluorescence-labeled proteins in buffered water and human midcycle cervical mucus

	Molecular mass	D_{water}*	d‡	D_{mucus}§	(D_{mucus}/D_{water})
	(Da)	(10^{-7} cm^2/s)	(nm)	(10^{-7} cm^2/s)	
Fluorescein	300	57 ± 0.7	0.86	53	0.94
Polypeptide	1,200	21 ± 5.0	2.4	18 ± 0.21	0.89 ± 0.22
Lactalbumin	14,500	13 ± 3.4	3.8	12 ± 4.9	0.91 ± 0.45
Ovalbumin	45,000	8.7 ± 0.7	5.6	8.8 ± 1.8	1.0 ± 0.23
Fc fragment	50,000	8.4 ± 0.38	5.8	6.7 ± 0.65	0.80 ± 0.085
F(ab) fragment	50,000	8.3 ± 1.0	6.0	6.7 ± 0.47	0.81 ± 0.11
BSA	68,000	8.3 ± 1.7	6.0	5.7 ± 0.35	0.68 ± 0.14
F(ab')$_2$ fragment	100,000	6.7 ± 0.24	7.2	4.4 ± 0.23	0.65 ± 0.041
Human IgA	150,000	5.2 ± 0.3	9.4	5.1 ± 0.14	0.99 ± 0.069
Human IgG	150,000	4.4 ± 1.3	11	2.9 ± 1.2	0.66 ± 0.34
Human IgM	970,000	3.2 ± 1.4	15	2.8 ± 1.2	0.89 ± 0.54
Human S-IgA¶	—	1.7 ± 0.14	28	0.49 ± 0.31	0.29 ± 0.18

* Diffusion coefficient in water at 25°C determined by FIP.

‡ Diameter 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 (0.0089 g/cm-s at 25°C), and D_{water} is the diffusion coefficient in water at 25°C determined by FIP.

§ Diffusion coefficient in human midcycle cervical mucus at 25°C determined by FIP.

¶ Aggregates of 400,000-Da S-IgA monomers.

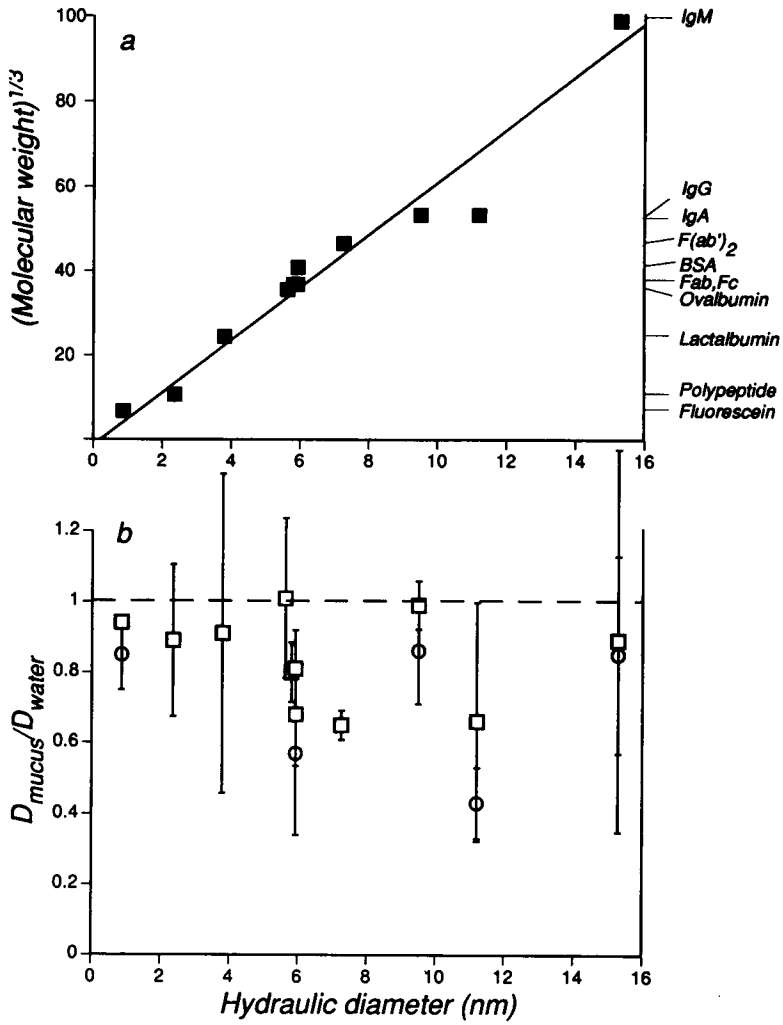

FIGURE 2 (*a*) The cube root of molecular weight (published value) as a function of hydraulic diameter (■) for each of the fluorescent proteins. The hydraulic diameters were calculated from the diffusion coefficients in water measured by FIP, as described in Table 1. (*b*) Reduced diffusion coefficient in mucus, as determined by FIP (□) and by FPR (○) after release from a test slab of polymer, is indicated for the same fluorescence-labeled proteins. Error bars indicate the standard deviation for three to five measurements.

spherical molecules varies with the cube root of the molecular weight (M_w), the molecular weight dependence of the diffusion coefficient is frequently approximated with the following power law expression (Polson, 1950):

$$D_{water} = A(M_w)^{-1/3} \quad (2)$$

While other equations for correlating protein diffusion coefficients have been evaluated (for a review of some typical correlations, see Tyn and Gusek, 1990), none are significantly better than Eq. 2. Equation 2 compared favorably to the literature data for protein diffusion and to our data (Fig. 1). Therefore, to approximate the size of each diffusing molecule, the hydraulic diameter for each of the fluorescence-labeled proteins was calculated from Eq. 1 (Table 1).

Polyacrylamide/agarose gel electrophoresis of our S-IgA preparations consistently revealed a broad protein band between 400,000 and 4,000,000 Da (Padgett, Whaley, and Cone, unpublished data). Therefore, we were not surprised to find that S-IgA diffused much more slowly than expected, based on the 400,000-Da molecular mass of the dimer; the measured rate of diffusion suggests a 28-nm diameter particle, which would have a molecular mass of $\sim 10^7$ Da (Table 1). Since all of our S-IgA preparations exhibited significant aggregation, which was consistent with the slow diffusion observed in water, we did not include measurements of S-IgA diffusion in the figures.

The diffusion coefficient of the fluorescence-labeled proteins in human midcycle cervical mucus was also determined by FIP after release from an EVAc polymer (Table 1). For all proteins examined, the rate of diffusion in mucus was slightly slower than the rate of diffusion in buffered water; reduced diffusion coefficients were determined by dividing the diffusion coefficient in mucus by the diffusion coefficient in water. This reduced diffusion coefficient was near unity for most of the proteins tested (Fig. 2 *b*), suggesting that diffusion of the proteins was only slightly slowed in mucus. For comparison, the fluorescent proteins were arranged in order of increasing hydraulic diameter, which is also indicated (Fig. 2 *a*). To confirm the values determined by FIP, reduced diffusion coefficients for certain probes (fluorescein, BSA, IgG, IgA, and IgM) were also determined by FPR. In all cases, the reduced diffusion coefficients obtained by FIP and FPR did not differ significantly (Fig. 2).

Reduced diffusion coefficients within a fibrillar collagen gel (1.0 mg/ml) and gelatin (100 mg/ml) were determined for fluorescein and selected fluorescent proteins (Table 2 and

TABLE 2 Reduced diffusion coefficient for fluorescein and fluorescence-labeled protein diffusion through gels of biopolymers (mucus, collagen, and gelatin)

	$(D_{collagen}/D_{water})^*$	(D_{mucus}/D_{water})	$(D_{gelatin}/D_{water})^‡$
Fluorescein	0.94 ± 0.1	0.94	0.50 ± 0.02
BSA	0.83 ± 0.4	0.68 ± 0.14	0.11 ± 0.03
Human IgG	0.98 ± 0.2	0.66 ± 0.34	0.10 ± 0.01
Human IgM	1.0 ± 0.5	0.89 ± 0.54	0.17 ± 0.09
Human S-IgA	0.56 ± 0.2	0.29 ± 0.18	0.20 ± 0.1

* Diffusion coefficient in 1.0 mg/ml collagen divided by the diffusion coefficient in buffered water determined by FPR at 25°C.

‡ Diffusion coefficient in 100 mg/ml gelatin divided by the diffusion coefficient in buffered water determined by FPR at 25°C.

Fig. 3). For each molecule, the reduced diffusion coefficient varied predictably in the three gel systems:

$$\frac{D_{gelatin}}{D_{water}} < \frac{D_{mucus}}{D_{water}} < \frac{D_{collagen}}{D_{water}}.$$

Diffusion coefficients for these same probes were also determined in dilute gelatin (1.0 mg/ml); the reduced diffusion coefficients in this case were essentially the same as in the collagen gel (data not shown). Within each gel, the reduced diffusion coefficient tended to decrease with increasing hydraulic diameter and also with increasing gel density.

Human midcycle cervical mucus is a highly hydrated gel containing mucin fibers (Fig. 4). The average mesh size within the gel was difficult to determine from scanning electron micrographs due to the three-dimensional nature of the samples and the potential for shrinkage during drying. These micrographs suggest, however, that the ~30-nm diameter mucin fibers form a loose mesh with an approximate spacing of 100–200 nm. Gravimetric analysis of three representative mucus samples yielded ~95% water by weight, ~5% total solids, and 2% solids of size greater than 6000 Da. At this level of magnification, the mucus structure appeared similar to the structure of collagen gels (0.1–1.0 mg/ml), except that the diameter of collagen fibers was substantially larger (~150 nm) (Parkhurst and Saltzman, 1992).

For fibers arranged on a cubic lattice mesh, the interfiber spacing, δ_{max}, is related to the fiber diameter, d_f, by:

$$\delta_{max} = d_f\sqrt{\frac{3\pi}{4V_f}} \qquad (3)$$

where V_f is the volume fraction of fibers in the gel. Although the interfiber spacing decreases with increasing fiber concentration, its dependence on concentration is weak:

$$\delta_{max} \propto 1/\sqrt{\text{concentration}}.$$

Since a cubic lattice arrangement is highly organized, with a regular pattern that maximizes the distance between individual fibers, Eq. 3 provides an upper bound on the interfiber spacing. The structure of the gels is actually a random arrangement of randomly oriented rods. In this complex environment, the geometry can be described by a probability function, P_d, giving the probability that a sphere, placed randomly within the matrix, will not overlap any of the randomly oriented rods. The probability that a randomly inserted sphere of diameter d_p will not intersect with any of the fibers is given by (Fanti and Glandt, 1990a,b):

$$P_d = \exp\left\{-\frac{\pi}{4}L_f(d_p + d_f)^2\right\} = \exp\left\{-\frac{V_f}{d_f^2}(d_p + d_f)^2\right\} \qquad (4)$$

where L_f is the length of fiber per volume, so that $L_f\pi(d_f/2)^2 = V_f$. Using this probability function, the interfiber spacing for randomly oriented fibers, δ_{ran}, can be approximated as the particle diameter, d_p, that gives a 50% probability of fitting within the lattice. This assumption yields the following

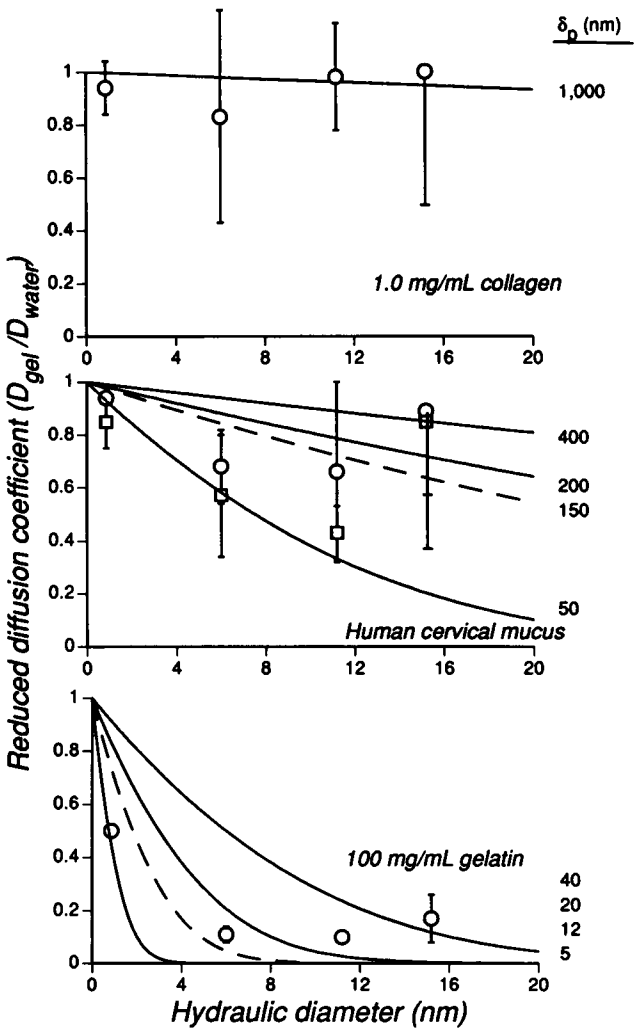

FIGURE 3 Reduced diffusion coefficients as a function of hydraulic diameter for fluorescein, BSA, IgG, and IgM in (*top*) collagen gel (1.0 mg/ml), (*center*) human cervical mucus, and (*bottom*) gelatin (100 mg/ml). Diffusion coefficients were determined by FIP (□) and by FPR (○). The *dashed lines* indicate the best fit of Eq. 6 to the experimental data with the average pore size of the gel (δ_p) as an adjustable parameter: $(\delta_p)_{collagen}$ ~1000 nm, $(\delta_p)_{mucus}$ = 150 nm, and $(\delta_p)_{gelatin}$ = 12 nm. The *solid lines* indicate solutions to Eq. 6 with other reasonable values of δ_p; they are provided to indicate how the predicted reduced diffusion coefficient varies with pore size.

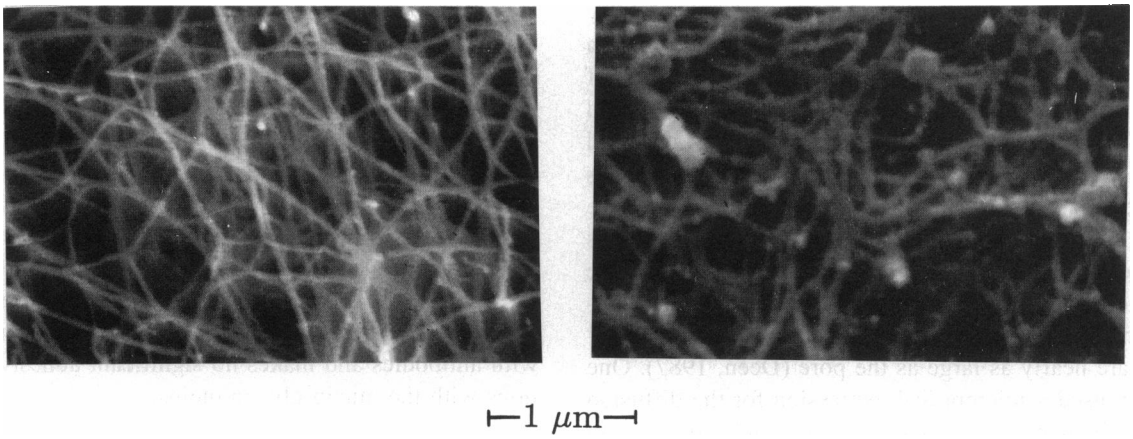

FIGURE 4 Scanning electron micrographs showing the structure of human midcycle cervical mucus. The two samples were obtained from two different subjects and prepared for scanning electron microscopy by fixation with glutaraldehyde, dehydration with ethanol, and critical point drying with carbon dioxide.

TABLE 3 Characteristic geometric features of collagen and mucin gels

	V_f*	d_f‡	δ_{max}§	δ_{ran}¶	δ_p‖
	(cm^3/cm^3)	(nm)	(nm)	(nm)	(nm)
Collagen gel (1.0 mg/ml)	0.00074	150	8500	4400	>1000
Human cervical mucus	0.015	30	380	170	150
Gelatin (100 mg/ml)	0.074	~1.5	8.5	3.1	12

* Calculated from mass fraction of fibers by assuming a specific volume of 0.74 cm^3/g for the fibers (Lehninger, 1975).

‡ Fiber diameter determined by SEM for collagen and mucus (see Fig. 4 and Parkhurst and Saltzman, 1992). Gelatin fibers could not be visualized by our SEM techniques, so d_f was approximated as the diameter of tropocollagen molecules (Alberts et al., 1989); the actual glycosylated fibers in gelatin may be somewhat larger than this, which would slightly increase the calculated fiber spacings for cubic and random lattice arrangements.

§ Maximum interfiber spacing, approximated by assuming cubic lattice arrangement (Eq. 3).

¶ Probable interfiber spacing, approximated by assuming random fiber arrangement (Eq. 4).

‖ Inferred interfiber spacing obtained by fitting Eq. 6 to measured diffusion coefficients (Fig. 3).

estimate:

$$\delta_{ran} = d_f\left[\sqrt{\frac{\ln 2}{V_f}} - 1\right] \qquad (5)$$

Table 3 shows some representative parameters describing the geometry of collagen and mucin gels.

DISCUSSION

Fluorescence-labeled proteins were slowly released from EVAc matrices. As demonstrated in previous studies, the rate of protein release from the matrix depends on protein molecular weight (Saltzman and Langer, 1989), size and mass fraction of dispersed protein particles (Saltzman and Langer, 1989), composition of the protein particles (Sherwood et al., 1992), and polymer molecular weight within the matrix (Saltzman et al., 1993). In the present study, the ability of proteins released from EVAc matrices to diffuse through unstirred layers of water and mucus was evaluated. When proteins were released from an EVAc matrix into buffered water, the diffusion coefficient in the water varied as expected with molecular weight of the protein (Fig. 1) and closely matched, quantitatively, a large body of previous observations based on many other methods. However, one of the proteins tested, S-IgA, diffused slower than expected based on its nominal molecular weight (Table 1), and we subsequently found that our preparations of S-IgA were aggregated.

Surprisingly, all of the proteins diffused nearly as rapidly in mucus as in water (Table 1 and Fig. 2 *b*). Even the aggregated S-IgA diffused only 3 times more slowly in mucus (Table 1). The other antibodies—IgG, IgA, and IgM—diffused at approximately the same rate in water and mucus. This result is contrary to the general impression that mucus forms a significant barrier to the diffusional transport of macromolecules. To our knowledge, however, these observations are the first to be made on fresh, unmodified mucus samples and are not subject to the significant problems associated with previous methods, as discussed above. Of course, rates of diffusion in less hydrated mucus, such as non-midcycle cervical mucus, might be slower than the diffusion measured here. However, since the interfiber spacing depends only weakly on hydration,

$$\delta_{max} \propto 1/\sqrt{\text{concentration}}$$

(see Eqs. 3 and 5), we expect that diffusion rates in other mucus samples will not differ markedly from those reported here.

It is difficult to predict the rate of diffusion of an arbitrary solute through a fibrous gel. Considerable progress has been made using free-volume theories to describe the diffusion of small solutes in dilute polymer solutions or gels (Vrentas and Duda, 1979). Although free-volume theories have been used to describe the diffusion of small molecules in mucus (Peppas et al., 1984), they are not as appropriate for large diffusing species like proteins, which are nearly as large as the characteristic distance between fibers in the gel. On the other hand, the diffusion of spherical solutes through cylindrical pores can be described quite accurately, even for solutes that are nearly as large as the pore (Deen, 1987). One commonly used semiempirical expression for the diffusion of solutes in porous materials was developed by Renkin (Renkin, 1954) to describe the diffusion of proteins in cellulose membranes. According to Renkin, the reduced diffusion coefficient is given by:

$$\frac{D_{ig}}{D_{iw}} = \left(1 - \frac{d}{\delta_p}\right)^2 \cdot \left[1 - 2.104\left(\frac{d}{\delta_p}\right) + 2.09\left(\frac{d}{\delta_p}\right)^3 - 0.95\left(\frac{d}{\delta_p}\right)^5\right] \quad (6)$$

where D_{ig} is the diffusion coefficient of solute i in the gel, D_{iw} is the diffusion coefficient of the solute in water, and δ_p is the average pore diameter of the material. This equation has been derived by calculating the drag on a spherical particle within a cylindrical pore under Stokes flow conditions, by assuming that the spherical particles are confined to the center line of the pore (Anderson and Quinn, 1974). While more sophisticated models for hindered diffusion in liquid pores are available (see (Deen, 1987) for an excellent review), the expression shown in Eq. 6 captures most of the important features. While some promising new techniques for using hydraulic permeabilities as the basis for estimating reduced diffusion coefficients in gels are available (Phillips et al., 1989), hydraulic permeabilities were not available for our samples.

Fig. 3 shows the reduced diffusion coefficient for a number of probes in collagen gel, cervical mucus, and gelatin (data reproduced from Table 2). The best value for the average pore size within the gel, δ_p, was obtained by comparing Eq. 6 with the experimentally measured diffusion coefficients in each gel; best fit values for δ_p are indicated in Table 3. These values are near the average pore sizes estimated by geometric arguments: $(\delta_p)_{collagen} > 1000$ nm, $(\delta_p)_{mucus} \sim 100$ nm, and $(\delta_p)_{gelatin} \sim 10$ nm. These pore sizes are also consistent with our electron microscopic evidence (Fig. 4) and with previous fine structural studies of cervical mucus (Yudin et al., 1989).

Large proteins diffuse readily through the water-filled interstitial spaces in midcycle cervical mucus. Since they diffuse readily, antibodies within the mucus—either secreted or topically applied (Radomsky et al., 1992)—will distribute rapidly throughout the mucus secretions and the mucus will not hinder the ability of antibodies to diffuse to pathogen surfaces. We appreciate that S-IgA, since it is abundant within the mucus secretions, is likely to play a major role in mucosal immunity. Because our observations of S-IgA suggest that our preparations had undergone significant aggregation, the present study does not provide a definitive portrait of S-IgA diffusion in mucus. However, high aggregates of S-IgA did diffuse rapidly through mucus (Table 1), suggesting that S-IgA dimers will diffuse even more readily and, moreover, our observations suggest that viral particles of about the same size as our S-IgA aggregates (30 nm) will encounter little resistance to diffusion in cervical mucus, provided the virus is not coated with antibodies and makes no significant adhesive interactions with the mucin glycoproteins.

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Note Added in Proof—Since final acceptance of this manuscript for publication, we became aware of a paper (Henry, B. T., J. Adler, S. Hibberd, M. S. Cheema, S. S. Davis, and T. G. Rogers. 1992. Epi-fluorescence microscopy and image analysis used to measure diffusion coefficients in gel systems. *J. Pharm. Pharmacol.* 44:543–549) that describes a similar technique for measuring fluorescent dextran diffusion in pig intestinal mucus. The measurements by Henry et al. are consistent with the measurements reported here.

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