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High-Molecular-Weight Polyethylene Glycol Prevents Lethal Sepsis Due to Intestinal *Pseudomonas aeruginosa*

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Background & Aims: **During stress, erosion of protective intestinal mucus occurs in association with adherence to and disruption of the intestinal epithelial barrier by invading opportunistic microbial pathogens. The aims of this study were to test the ability of a high-molecular-weight polyethylene glycol compound, polyethylene glycol 15–20, to protect the intestinal epithelium against microbial invasion during stress.** ***Methods:*** **The ability of polyethylene glycol 15–20 to protect the intestinal epithelium against the opportunistic pathogen *Pseudomonas aeruginosa* was tested in cultured Caco-2 cells. Bacterial virulence gene expression, bacterial adherence, and transepithelial electrical resistance were examined in response to apical inoculation of *P. aeruginosa* onto Caco-2 cells. Complementary in vivo studies were performed in a murine model of lethal sepsis due to intestinal *P. aeruginosa* in which surgical stress (30% hepatectomy) was combined with direct inoculation of *P. aeruginosa* into the cecum.** ***Results:*** **High-molecular-weight polyethylene glycol (polyethylene glycol 15–20) conferred complete protection against the barrier-dysregulating effects of *P. aeruginosa* in Caco-2 cells. Intestinal application of polyethylene glycol 15–20 in stressed mice protected against the lethal effects of intestinal *P. aeruginosa*. Mechanisms of this effect seem to involve the ability of polyethylene glycol 15–20 to distance *P. aeruginosa* from the intestinal epithelium and render it completely insensate to key environmental stimuli that activate its virulence.** ***Conclusions:*** **High-molecular-weight polyethylene glycol has the potential to function as a surrogate mucin within the intestinal tract of a stressed host by inhibiting key interactive events between colonizing microbes and their epithelial cell targets.**

The ability of the intestinal epithelium to resist the adhesion of invading microbes is generated, in large part, by the presence of variously charged mucins comprising the so-called unstirred water layer of the intestinal mucosa. Recent studies on the biochemistry and physiology of intestinal mucins suggest that these compounds protect the intestinal epithelium against microbial invasion by affecting key physicochemical surface properties of both the bacteria and epithelial cells such that a net repulsive force is generated.[1] Under homeostatic conditions, many antagonistic and opposing forces act to distance bacteria from the epithelial surface: these include van der Waals forces, surface electric charge, and hydrophobicity. To a large degree, various charged mucins within the unstirred water layer of the intestinal epithelium seem to play a key role in the governance of such forces.[2] For example, the removal of intestinal mucus with specific detergents results in significant adherence of indigenous bacteria to the intestinal epithelium in association with a significant defect in intestinal permeability.[3] Similarly, the introduction of a systemic stress, such as the injection of lipopolysaccharide[3] or restraint stress,[4] results in depletion of the mucus layer coupled with marked alterations in intestinal permeability and barrier function to invading indigenous bacteria. Finally, an additional role for the protective effect of mucus against bacterial invasion of the intestinal epithelium is suggested by the observation that preincubation of pathogenic strains of bacteria with intestinal mucus significantly alters their surface characteristics and reduces their ability to adhere to cultured intestinal epithelial cells.[5,6] Taken together, these studies strongly suggest that stress-induced depletion of intestinal mucus alone could promote bacterial-mediated disorders of the intestinal tract by eroding an important component of

Abbreviations used in this paper: AFM, atomic force microscopy; cfu, colony-forming units; DIC, differential interference contrast; EGFP, enhanced green fluorescent protein; HSL, homoserine lactone; LDH, lactate dehydrogenase; PA-I, *Pseudomonas aeruginosa* lectin/adhesin I; TEER, transepithelial electrical resistance.

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the innate intestinal defense system that has a unique function within the host/pathogen interface.

Polyethylene glycol compounds (PEGs) may be ideally suited to act as surrogate mucin compounds within the intestinal tract of a stressed host on the basis of their hydrophobic/hydrophilic properties. Depending on their molecular weight, linear conformation, and concentration, PEGs can anchor to innate or living surfaces and exert major changes in surface electric charge, hydrophobicity, and van der Waals forces to approaching proteins. The precise mechanism for these effects is yet to be fully understood; however, PEG compounds display unique behavior on biological surfaces as the ability of these polymers to adopt higher-order intrachain structures changes their conformational freedom and, hence, their repellant effect to approaching proteins.[7] The completely inert and nontoxic nature of these compounds makes them highly attractive as therapeutic agents to chemically shield the intestinal epithelium during clinical disorders in which epithelial barrier function to highly invasive pathogens is compromised.

In this study, we sought to determine whether intraluminal application of a high-molecular-weight PEG compound (PEG 15–20 daltons) could functionally reestablish the mucus barrier of the intestinal epithelium and protect against a highly toxic and lethal opportunistic pathogen expressing a high adherence phenotype under conditions of catabolic stress. Our laboratory has developed a novel model of lethal gut-derived sepsis by exposing normal mice to catabolic stress and short-term starvation (24–48 hours of H_2O only) in combination with the direct introduction of *Pseudomonas aeruginosa* into the cecum.[8] This model results in clinically severe sepsis and a 100% mortality rate at 48 hours. Lethality due to intestinal *P. aeruginosa* in this model is dependent on the in vivo expression of a key virulence determinant in *P. aeruginosa,* the *P. aeruginosa* lectin/adhesin I (PA-I), which facilitates adhesion of the organism to the intestinal epithelium. We have also shown that the PA-I lectin plays a major role in the pathogenesis of intestinal *P. aeruginosa* by creating a defect in the intestinal epithelial barrier to 2 lethal cytotoxins of this organism: exotoxin A and elastase.[9] The critical role of PA-I expression in lethal gut-derived sepsis due to intestinal *P. aeruginosa* is suggested by our previous work showing that null mutant strains of *P. aeruginosa* lacking PA-I are completely apathogenic in this model. We proposed that this model would be ideally suited to test the ability of PEG to prevent gut-derived sepsis caused by *P. aeruginosa* as both a specific bacterial virulence marker and an epithelial barrier function that could be tracked through the course of infection. Therefore, the aims of this study were to determine the efficacy and mechanism of action of high-molecular-weight PEG (PEG 15–20) as a method to prevent lethal gut-derived sepsis in mice due to intestinal *P. aeruginosa*.

Materials and Methods

Bacterial Strains, Epithelial Cell Lines, and Polyethylene Glycol Solutions

P. aeruginosa strain 27853 (PA27853; American Type Culture Collection, Manassas, VA) is a nonmucoid clinical strain originally isolated from a blood culture. All experiments were performed with Caco-2/C2bbe, or C2, cells (passage 51–68) and were obtained as a generous gift of Dr. Mark Mooseker (Yale University, New Haven, CT). C2 cells are Caco-2 subclones that are highly differentiated intestinal epithelial cells.[10] Two PEGs of different molecular weights were purchased from Sigma (St. Louis, MO): PEG 3350 (catalog no. P4338) and PEG 15,000–20,000 (catalog no. P2263). They are designated as PEG 3.35 and PEG 15–20, respectively.

PA27853/Enhanced Green Fluorescent Protein and PA27853/PLL–Enhanced Green Fluorescent Protein Constructs

To develop stable PA-I reporter strains in PA27853, strains were constructed to PA27853/PLL-enhanced green fluorescent protein (EGFP) by using the *E. coli* shuttle vector as outlined[11] in Figure 1. The final constructs were termed PA27853/PLL-EGFP to denote them as constructs using the original strain PA27853 with the Promoter of the PA-I Lectin plus the PA-I Lectin gene itself fused to EGFP protein.

Mouse Model of Gut-Derived Sepsis

Female BALB/c mice were anesthetized and subjected to hepatectomy as reported.[7] All mouse experiments were approved by the Animal Care and Use Committee of the University of Chicago. A 30% bloodless excision of the liver along the floppy left lobe was performed by using a surgical electrocautery. Control mice underwent manipulation of the liver without hepatectomy. In all mice, the cecal lumen was accessed by direct needle puncture into the base. One milliliter of 0.9% NaCl, 5% PEG 3.35, or 2.5%–5% PEG 15–20 was injected retrograde into the small bowel. Next, 200 μL of 10^7 colony-forming units (cfu) per milliliter of PA27853 diluted in 0.9% NaCl, 10% PEG 3.35, or 2.5%–10% PEG 15–20 was injected into the cecum through the same needle. The puncture site was tied off with a silk suture and swabbed with alcohol. Mice were returned to their cages, given H_2O only, and followed up for mortality for 48 hours. Reiterative experiments were performed after infection whereby mice (n = 5) were gavaged with water, 5% PEG 3.35, or 5% PEG 15–20 (750 μL per os via feeding tube) at 4 and 8 hours after hepatectomy and cecal injection of PA27853. Mice were al-

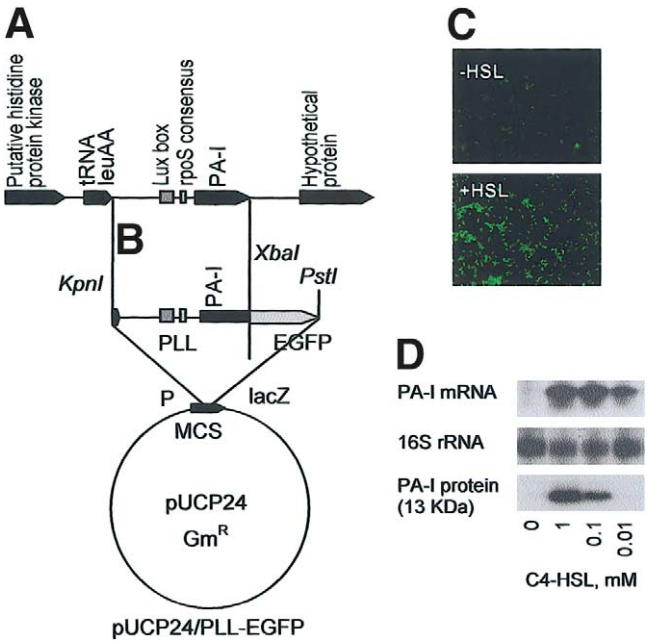

Figure 1. Construction of PA-I reporter plasmid pUCP24/PLL–EGFP. (*A*) The entire PA-I gene and upstream regulator region in *P. aeruginosa* strain PA27853. A lux box–type element of quorum-sensing signaling system and RpoS (σS) consensus are shown. (*B*) Fusion construct: the pll region (440 base pairs [bp] upstream plus 366 bp of the entire PA-I [minus stop codon]) was amplified from PA27853 genomic DNA. The 440 bp upstream included the C-terminal of tRNALeu followed by the regulator region of PA-I. The restriction sites *Kpn*I and *Xba*I were inserted by using the following respective primers: forward, 5′-GGTACCCCGGTTCGACCCCGGCTCCGG-3′; and reverse, 5′-TCTAGAGGACTGATCCTTTCCAATAT-3′. The Egfp gene coded for green fluorescent protein was amplified with the pBI-EGFP plasmid (Clontech, Palo Alto, CA) as a template. *Xba*I and *Pst*I restriction sites were introduced by using the following respective primers: forward, 5′-TCTAGAACTAGTGGATCCCCGCGGATG-3′; and reverse, 5′-GCAGACTAGGTCGACAAGCTTGATATC-3′. pll was fused with egfp via the *Xba*I restriction site and ligated into pUCP24 digested with *Kpn*I and *Pst*I to create pUCP24/PLL–EGFP. Reporter plasmid pUCP24/PLL–EGFP was electroporated into PA27853 electrocompetent cells to create reporter strain PA27853/PLL–EGFP. PA27853/PLL–EGFP constructs were selected on the basis of gentamicin (100 μg/mL) resistance and fluorescence induced by the cognate quorum-sensing signaling molecule for PA-I, C4-homoserine lactone (C4-HSL; 0.1 mmol/L). (*C*) PA27853/PLL–EGFP grown in the presence of C4-HSL showed significant fluorescence. (*D*) C4-HSL concentration-dependent induction of PA-I expression estimated by Northern blot and immunoblot.

lowed free access to water only and were then observed for signs of sepsis and mortality for 48 hours. Quantitative cultures of feces, vigorously washed cecal mucosa, liver, and blood were performed on *Pseudomonas* isolation agar as previously described.[8] Mice who seemed moribund were killed before natural death by exsanguination before the tissue harvest.

PA-I Expression In Vivo

Reporter strain PA27853/PLL–EGFP was used in the mouse model of gut-derived sepsis to follow the expression of PA-I in vivo. Two hundred microliters of 10^6 cfu per milliliter of PA27853/PLL–EGFP was introduced into the cecum of sham-operated and 30% hepatectomy mice with or without 10% PEGs. After 24 hours, the mice were killed, and cecal contents (feces) were collected and mixed into a crude liquid/particulate fraction in phosphate-buffered saline (PBS). The liquid portion of the mixture was harvested and spun at 5000*g* for 10 minutes. The cecum was wrapped with OCT medium (IMEB, Sakura, Japan), frozen in liquid nitrogen, and cryosectioned at 5-μm intervals. The processed fecal samples and cecal mucosal sections were viewed under a Zeiss Axioplan fluorescence microscope (Thornwood, NY) by using differential interference contrast (DIC) and GFP filters. To quantitate fluorescence of the processed fecal samples, samples were resuspended in sonication buffer (50 mmol/L NaH_2PO_4, 10 mmol/L Tris HCl, pH 8.0, and 200 mmol/L NaCl) and disrupted with a Sonic Dismembrator (Fisher, Chicago, IL). Debris was removed by centrifugation while the fluorescence of the cytosolic fraction was measured with a Versafluor fluorometer (Bio-Rad, Hercules, CA). Protein concentration was routinely measured with the micro-bicinchoninic acid method (Pierce, Rockford, IL). Fluorescence was expressed as relative fluorescence units per microgram of protein.

Bacterial Adherence Assay

The number of bacteria that were associated with the epithelial monolayers was determined by using a previously published method.[12] Briefly, overnight cultures of *P. aeruginosa* were inoculated at 1:1000 dilution with or without 10% PEGs onto confluent Caco-2 cells and cocultured for 4 hours (37°C; 5% CO_2). Media were completely removed from the Caco-2 cell culture wells, and *P. aeruginosa* colony counts were determined by plating 10-fold dilutions on *Pseudomonas* isolation agar. Next, Caco-2 cells were washed with PBS twice and scraped into centrifuge tubes followed by extensive washing with PBS, spun down at 900*g* for 10 minutes, and homogenized in 0.5 mL of PBS. The number of adherent *P. aeruginosa* was determined by quantitative culture on *Pseudomonas* isolation agar. Colony counts were normalized to the protein concentration of homogenized Caco-2 cells. The total amount of *P. aeruginosa* was determined by collecting and combining both media and scrapped Caco-2 cells after co-incubation with PA27853.

Analysis of PA-I Expression In Vitro

Northern hybridization for PA-I messenger RNA (mRNA) was measured as previously described.[9] PA-I protein was measured by Western blotting as previously described.[9] Reporter strain PA27853/PLL–EGFP was used to quantitate PA-I expression by measuring the expression of the reporter gene EGFP. After they were grown in the presence of C4-homoserine lactone (HSL) and PEGs, PA27853/PLL–EGFP bacteria were washed with PBS and disrupted by sonication, and fluorescence of the cytosol fraction was measured as described previously.

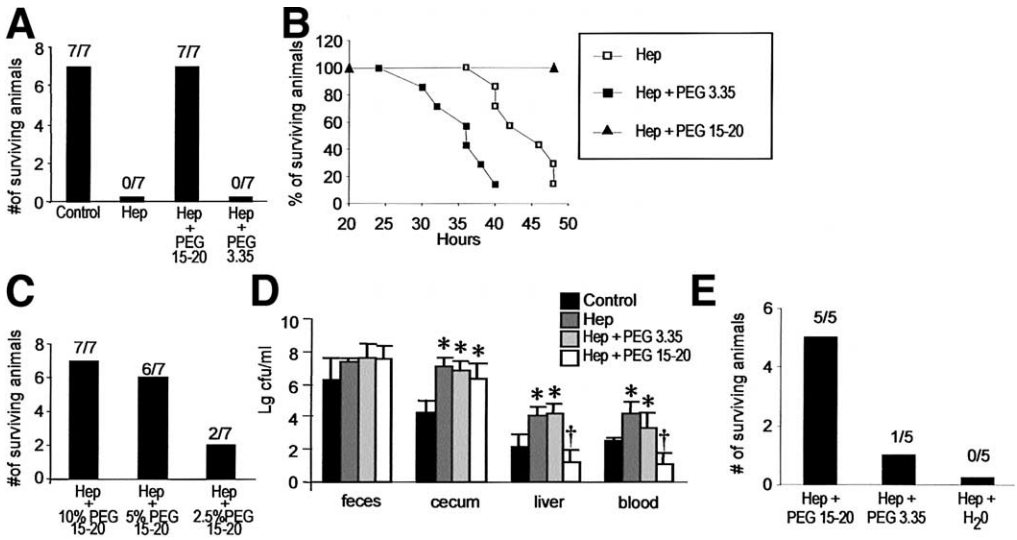

Figure 2. Mortality rates in mice at 48 hours subjected to either sham laparotomy or 30% surgical hepatectomy followed by direct injection of PA27853 into the cecum. Mice underwent a 30% bloodless left lobe hepatectomy (Hep) that was immediately followed by direct cecal injection of PA27853 (n = 7 per group). Control mice underwent sham laparotomy followed by injection of equal amounts of PA27853 into the cecum. (*A*) Protective effect of PEG 15–20 on mortality in mice after hepatectomy and direct cecal injection of PA27853 ($P < 0.001$; Fisher exact test). (*B*) Time course of mortality in mice undergoing hepatectomy and treated with PEG 3.35 or PEG 15–20. (*C*) Concentration-dependent protective effect of PEG 15–20. (*D*) Quantitation of bacterial cultures in cecal contents (feces), washed cecal mucosa (cecum), liver, and blood 24 hours after 30% surgical hepatectomy and direct cecal injection of PA27853. All data are mean ± SEM. Sum-rank analysis of variance showed a statistically significant increase in bacterial counts in the cecum, liver, and blood of mice after hepatectomy (*$P < 0.001$). A significant decrease (†$P < 0.05$) in liver and blood bacterial counts was observed in mice after hepatectomy treated with PEG 15–20 (Hep + PEG 15–20) compared with mice undergoing hepatectomy without PEG treatment (Hep). (*E*) The effect of PEG rescue therapy on mortality after hepatectomy. PEG administered as an oral solution 4 and 8 hours after infection protected against mortality after hepatectomy and cecal injection of PA27853 ($P <$ 0.01; Fisher exact test).

Influence of Polyethylene Glycol on Growth Characteristics and Membrane Integrity of *Pseudomonas aeruginosa*

Growth curves for PA27853 were generated by plating 10-fold dilutions of samples taken at different points of growth in the absence and presence of 10% PEGs. The activity of the housekeeping enzyme lactate dehydrogenase (LDH) was measured with a coupled diaphorase enzymatic assay by using a substrate mix from CytoTox 96 (Promega, Madison, WI).

Atomic Force Microscopy

PA27853 was cultured in tryptic soy broth with or without 10% PEGs for 4 hours. Cells were extensively washed with PBS, and 1 drop of the bacterial suspension was dried on the top of mica in blowing air for 10 minutes and imaged immediately. Imaging of the dried bacteria with tapping mode atomic force microscopy (AFM) was performed in air with a Multimode Nanoscope IIIA Scanning Probe Microscope (MMAFM; Digital Instruments, Woodbury, NY). Monolayered Caco-2 cells were treated with 10% PEGs for 4 hours and washed with PBS extensively. Imaging of the cells was performed in PBS by AFM without using an O-ring.

Electron Microscopy

PA27853 was grown overnight in tryptic soy broth with or without C4-HSL 1 mmol/L and 10% PEGs. Cells were diluted in NaCl 0.5 mol/L at 1:100 and stained with uranyl acetate before examination with a Philips CM 120 electron microscope (Tallahassee, FL).

The Effect of Polyethylene Glycol Solutions on the Dispersion/Clumping Pattern of *Pseudomonas aeruginosa*

PA27853/EGFP was used in the following experiment. One hundred microliters of PA27853/EGFP, with EGFP expressed under the *Plac* by 0.5 mmol/L isopropylthiogalactoside, was mixed with 1 mL of Caco-2 cell medium with or without 10% PEGs and poured into a 0.15-mm-thick dTC3 dish (Bioptech, Bulter, PA) with or without monolayered Caco-2 cells. The dispersion/clumping pattern of live bacterial cells was monitored with an Axiovert 100 TV fluorescence-inverted microscope (Zeiss) with DIC and GFP filters.[13] The 3-dimensional imaging software (Slidebook) from Intelligent Imaging Innovations (Denver, CO) was used to image the dispersion pattern in the *z* plane by using the GFP filter.

Results

PEG 15–20 protects against lethal gut-derived sepsis due to *P. aeruginosa* in mice after 30% hepatectomy. Direct cecal injection of PA27853 into mice who underwent a 30% surgical hepatectomy resulted in a

state of clinical sepsis with no survivors at 48 hours (Figure 2*A*). Mice undergoing sham laparotomy without hepatectomy (controls), similarly injected with *P. aeruginosa,* survived completely without any clinical signs of sepsis (Figure 2*A*). To determine the ability of PEG solutions to prevent mortality in this model, PA27853 cells were suspended in one of two 10% (wt/vol) solutions of PEG (PEG 3.35 or PEG 15–20). PEG 3.35 was chosen because it represents the molecular weight of PEGs that are available for clinical use as intestinal cleansing agents (GoLYTELY; Braintree Inc., Braintree, MA).[14] We compared PEG 3.35 with high-molecular-weight PEG solutions (15–20 kilodaltons) because similar molecular weight PEGs have been reported to have a protective effect on the intestine by an unknown mechanism before transplantation.[15] Two hundred microliters of the bacterial/PEG suspension was introduced into the cecum by direct puncture. PEG 15–20 was completely protective against mortality in mice after hepatectomy, whereas PEG 3.35 delayed the onset of mortality, it had no protective effect at the 48-hour time point (Figure 2*A* and *B*). Mice treated with PEG 3.35 seemed septic (ruffled fur, chromodacryorrhea, and loose stools) before death, whereas mice treated with PEG 15–20 seemed completely healthy at 48 hours. Mice treated with the PEG solutions did not develop diarrhea. Mice undergoing hepatectomy that drank only water expelled 7–11 fecal pellets in 24 hours. As mice became septic, their stools became loose yet formed in pellets, and they did not develop diarrhea. PEG-treated mice had a small increase in stool frequency (compared with hepatectomy without PEG) to 14–21 pellets per 24 hours for PEG 3.35 and 18–25 pellets per 24 hours for PEG 15–20.

Dose–response experiments showed a 5% solution to be the minimal concentration of PEG 15–20 that was protective in this model (Figure 2*C*). Hepatectomy increased the adherence of PA27853 to the cecal mucosa and its translocation to the liver and blood. Only PEG 15–20 decreased the translocation of PA27853 to the liver and blood, although neither PEG 3.35 nor PEG 15–20 decreased adherence of PA27853 to the cecum as assessed by quantitative culture alone (Figure 2*D*). Because these studies were performed by administering PEG directly into the cecum and small bowel simultaneously with the infectious inoculum, we sought to determine whether PEG could also protect mice when it was administered orally after the initial infection, as might occur clinically. Results from the rescue experiments, in which mice were gavaged with water, 5% PEG 3.35, or 5% PEG 15–20 at 4 and 8 hours after hepatectomy and cecal injection of PA27853, showed that 5 of 5 mice survived after oral gavage with PEG 15–20 and seemed completely healthy at the 48-hour time point. Only 1 of 5 mice survived in the PEG 3.35–treated group and 0 of 5 in the water-only–treated group (Figure 2*E*). All mice in these 2 groups seemed septic. Culture results of tissues were similar to the previous set of experiments and showed that orally gavaged PEG 15–20 attenuated the magnitude of *P. aeruginosa* dissemination to the liver and blood (data not shown).

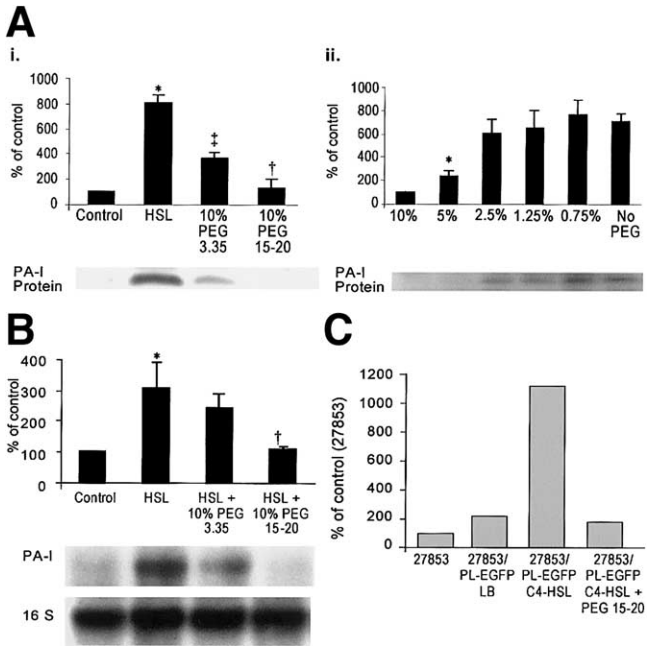

Figure 3. The inhibitory effect of PEGs on C4-HSL–induced PA-I expression. (*A*) Immunoblot. (*i*) Exposure of PA27853 to 1 mmol/L of the quorum-sensing signaling molecule C4-HSL resulted in a statistically significant increase (*$P < 0.00$; 1-way analysis of variance) in PA-I protein expression that was significantly inhibited (††$P < 0.01$ vs. HSL) in the presence of both 10% PEG 3.35 and 10% PEG 15–20, although to a greater degree with PEG 15–20. (*ii*) The minimum inhibitory concentration of PEG 15–20 on C4-HSL–induced PA-I expression was 5% (*$P < 0.001$; analysis of variance). (*B*) Northern blot. Exposure of PA27853 to 0.1 mmol/L of C4-HSL resulted in a statistically significant increase (*$P < 0.001$; analysis of variance) in PA-I mRNA expression that was inhibited with 10% PEG 15–20 (†$P <$ 0.01 vs. HSL). (*C*) Effect of PEGs on the expression of the reporter strain PL-EGFP/27853. Corroborative experiments showed that PL-EGFP/27853 was also insensitive to C4-HSL–induced fluorescence in the presence of PEG 15–20 in overnight experiments. All data are mean ± SEM.

PEG 15–20 inhibits virulence expression of *P. aeruginosa* in response to the quorum-sensing signaling molecule C4-HSL. A diverse array of virulence factors in *P. aeruginosa* is released in response to molecules of the quorum-sensing signaling system (for review, see Fuqua and Greenberg[16]). In particular, PA-I expression in *P. aeruginosa* is regulated by the transcriptional regulator rhamnolipids regulatory gene (*rhlR*) and its cognate activator C4-HSL.[17] We have previously shown that expo-

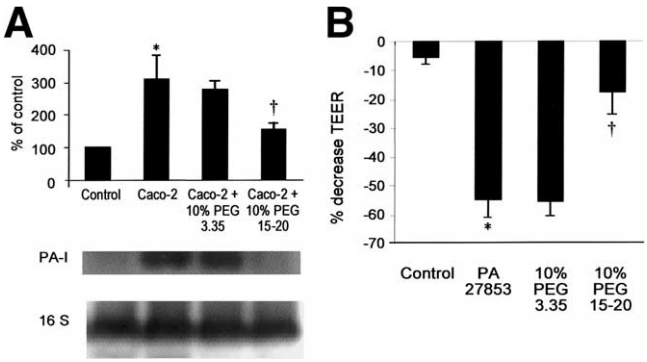

Figure 4. The effect of PEGs on PA-I expression induced by co-incubation with Caco-2 cells and their effect on *P. aeruginosa*–induced disruption of Caco-2 cell barrier function (TEER). (*A*) Caco-2 cells caused an increase in PA-I mRNA (*$P < 0.001$; analysis of variance) after a 4-hour exposure to bacterial cells of PA27853. PEG 15–20 resulted in a significant attenuation (†$P < 0.001$; 1-way analysis of variance) in Caco-2 cell–induced PA-I mRNA. (*B*) Apical inoculation of *P. aeruginosa* (PA27853) onto Caco-2 cells caused a significant (*$P < 0.001$) decrease in TEER. This effect was significantly attenuated (†$P < 0.01$ vs. PA27853) in the presence of PEG 15–20. All data are mean ± SEM of triplicate cultures (n = 7).

sure of PA27853 to C4-HSL results in a significant increase in PA-I expression.[18] Inhibition of both PA-I protein and PA-I mRNA was observed in PA27853 exposed to C4-HSL in the presence of 10% PEG 15–20 (Figure 3*Ai* and *B*). A significant inhibitory effect was also seen with PEG 3.35, although this seemed less at the level of mRNA. The minimum concentration of PEG 15–20 that inhibited C4-HSL–induced expression of PA-I protein was 5% (Figure 3*Aii*). The ability of PEG 15–20 to also render PA27853/PLL–EGFP unresponsive to C4-HSL was seen in overnight experiments (Figure 3*C*).

PEG 15–20 inhibits the virulence expression of *P. aeruginosa* in response to cultured intestinal epithelial cells and protects epithelial cells against *P. aeruginosa*–induced barrier disruption. Exposure of PA27853 to Caco-2 cells resulted in expression of PA-I mRNA within 4 hours of incubation (Figure 4*A*). This effect was significantly inhibited in the presence of PEG 15–20 (Figure 4*A*). As previously reported, PA27853 resulted in a profound decrease in the transepithelial electrical resistance (TEER) of confluent Caco-2 cells.[7,8] This effect was significantly attenuated in the presence of PEG 15–20, but not PEG 3.35 (Figure 4*B*).

PEG solutions do not affect the growth pattern of *P. aeruginosa*. Growth curves for PA27853 grown overnight in the presence of PEGs showed that bacterial quantity was unaffected by the presence of either PEG solution (Figure 5). The activity of a housekeeping enzyme involved in energy metabolism, LDH, was measured at the various time points from the exponential to the stationary phase of growth. No change in LDH activity in cytosolic extracts of PA27853 grown in the presence of PEGs was observed (data not shown).

Electron microscopy and AFM showed a distinct coating effect of PEG solutions on *P. aeruginosa* bacterial cells. Overnight exposure of PA27853 to C4-HSL 1 mmol/L changed the appearance of bacterial cells from that of a typical convoluted and rugged surface (Figure 6*A*; control) to a cellular appearance showing a flat surface without visible surface convolutions (Figure 6*A*; HSL). Bacteria exposed to overnight PEG 15–20 retained a morphological picture similar to that of strains not exposed to C4-HSL (Figure 6*A*; PEG 15–20). A visible electron halo was seen (*arrow*) around the surface of the bacterium in the presence of PEG 15–20. AFM of bacterial cell surfaces showed topographic changes at nanometer scales in the presence of the 2 polymers. PEG 3.35 created a smooth, uniform surface on bacterial cells, whereas the PEG 15–20 polymer appeared rugged on the surface of the bacterial cell (Figure 6*Biii*). Measurements of the height deflection of the polymer/bacterial surface by AFM showed an increase in the height of the bacterial/polymer surface with PEG 15–20 compared with PEG 3.35 (Figure 6*C*).

PEG 15–20 affects the clumping behavior of *P. aeruginosa* and its spatial relationship to the apical surface of cultured human intestinal epithelial cells. In media alone, bacteria are seen uniformly dispersed as planktonic *P. aeruginosa* cells on DIC and GFP images (Figure 7*A* and *B*). However, in the presence of Caco-2 cells, bacterial cells in media alone were adherent to the epithelial monolayers (Figure 7*C*). In the presence of 10% PEG 3.35, however, bacteria formed large mushroom-shaped clumps (Figure 7*A*) and adhered to the bottom of the well (Figure 7*B*). In the presence of Caco-2 cells, bacteria

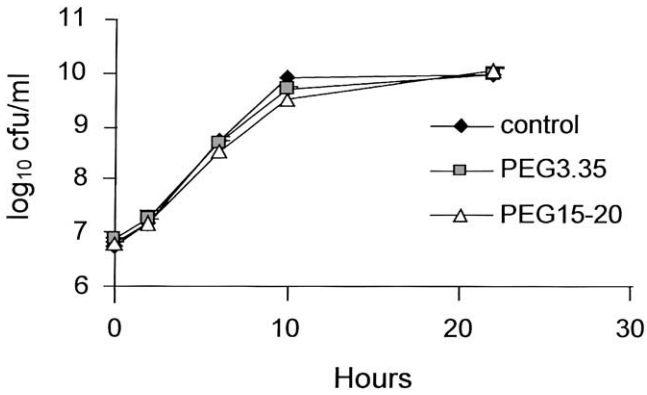

Figure 5. The effect of PEG solutions on growth of PA27853. PA27853 growth curves appeared identical in the presence of both 10% PEG solutions when compared with PEG-free tryptic soy broth medium. All data are mean ± SEM.

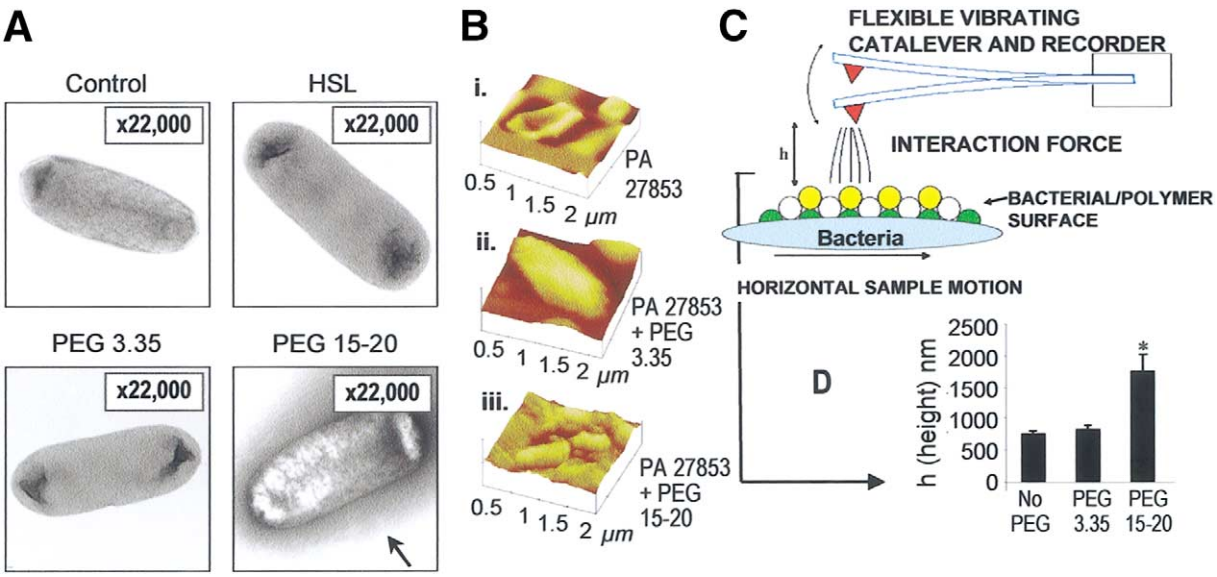

Figure 6. (*A*) Electron microscopy of individual bacteria cells exposed to C4-HSL in the presence and absence of 10% PEGs. Overnight exposure of bacterial cells to C4-HSL showed a loss of transparent surface convolutions typically seen in normal gram-negative bacteria as seen in control cells. Electron microscopy of bacterial cells exposed to 10% PEG 15–20, but not 10% PEG 3.35, showed retention of this transparent and convoluted surface, similar to control cells. In addition, PEG 15–20 created an electron halo effect around the perimeter of bacterial cells (*arrow*). To determine the nature of this halo effect, atomic force microscopy was used to examine bacterial cells. (*C*) Mechanism of atomic force microscopy (AFM). Besides recording topographic images, AFM generates force–distance measurements at a given *x, y* location as the cantilever deflection is recorded as a function of its vertical displacement in nanometers. (*B*) AFM images of PA27853 without PEG (*i*), in PEG 3.35 (*ii*), and in PEG 15–20 (*iii*) show distinct topographic images and height fluctuations. (*D*) PEG 15–20 shows the highest height of deflection ($*P < 0.01$; analysis of variance) off the bacterial/polymer surface. Data are mean ± SEM for multiple observations.

exposed to 10% PEG 3.35 were clumped and suspended in the order of 8 μm above the plane of the epithelial cells (Figure 7*C*). A similar, yet distinct, pattern was seen with PEG 15–20. For the first 0.5–1 hours of incubation, bacterial cells formed spider-shaped microclumps that were close to the bottom of the well (Figure 7*A* and *B*). Within several hours, spider leg–shaped microclumps occupied the entire space/volume of the medium (not shown). When co-incubated with Caco-2 cells, *P. aeruginosa* cells were elevated high above the plane of the epithelium (30–40 μm) in the presence of 10% PEG 15–20 (Figure 7*C*).

Experiments in which bacterial adhesion to Caco-2 cells was assessed as a ratio of adherent to nonadherent PA27853 cells showed that PEG 15–20 resulted in a greater concentration of bacteria remaining in the culture media. No difference in bacteria adherent to Caco-2 cells was observed in these experiments (Table 1).

To determine whether the expected up-regulation of PA-I in *P. aeruginosa* introduced into the cecum of mice undergoing hepatectomy could be suppressed in the presence of PEG solutions, the reporter strain PA27853/PLL–EGFP was injected into the cecum of mice after surgical hepatectomy and retrieved 24 hours later by harvesting and processing cecal contents (feces) and cecal mucosal tissues. Hepatectomy resulted in an increase in PA-I expression in both luminal bacteria (Figure 8*A* and *B*) and mucosally associated bacteria (Figure 8*C*). PEG 15–20 inhibited fluorescence in PA27853/PLL–EGFP in both luminal and mucosally associated bacteria (Figure 8*A–C*).

Discussion

In this study, the ability of PEG 15–20 to protect the intestinal epithelium against an opportunistic colonizing pathogen was assessed. Specifically, the ability of PEG to inactivate the lethal effects of intestinal *P. aeruginosa,* one of the more common causes of fatal gut-derived sepsis in an immunocompromised host, was tested.[19–21] Data from this show that there are striking effects of

Table 1. Bacterial Counts (mean ± SD) in Media and in Caco-2 Cell Preparations After Apical Exposure of Cell Cultures to 10^7 cfu/mL of *P. aeruginosa* 27853 for 4 Hours

Group (n = 5)	Total (*log cfu/mL*[a])	Media (*log cfu/mL*)	Caco-2 cells (*log cfu/mL*[a])
Control	6.87 ± 0.16	5.43 ± 0.17	6.15 ± 0.74
PEG 3.35	6.94 ± 0.21	5.75 ± 0.17	6.46 ± 0.60
PEG 15-20	7.05 ± 0.12	6.56 ± 0.30[b]	6.76 ± 0.70

[a]Values adjusted to 100 mg protein.
[b]$P < 0.05$ compared with controls.

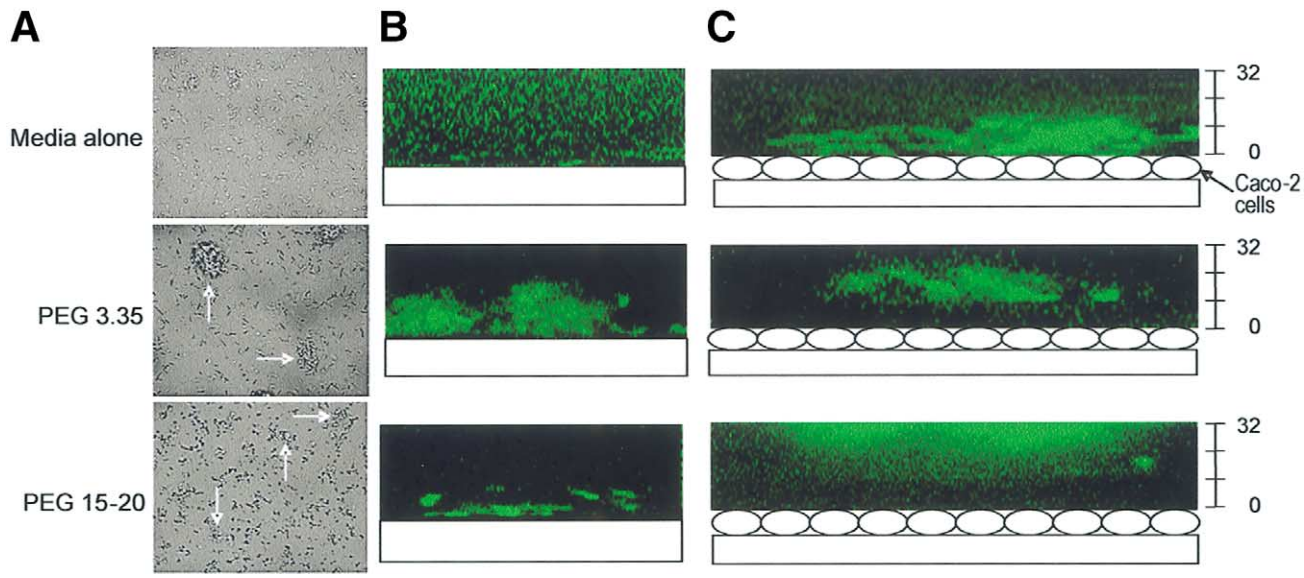

Figure 7. The effect of PEG solutions on bacterial clumping patterns and spatial orientation of bacteria in relation to Caco-2 cell surface. Column A shows overhead images of bacteria grown in Caco-2 cell high-glucose Douglas minimum Eagels media without epithelial cells. Columns B and C show *z*-plane reconstructions of multiple stacked images of PA27853/EGFP in the absence (*B*) and presence (*C*) of Caco-2 cells. Images in column A show that PEG 3.35 induces large clump formation in bacteria, whereas PEG 15–20 induces a distinct microclumping pattern (*arrows*). Fluorescent images in column B show that in media alone without Caco-2 cells, bacteria remain uniformly dispersed throughout the culture well, whereas in the presence of PEG 3.35 or PEG 15–20, bacteria form clumps and fall to the bottom of the culture well. Column C shows that the presence of the Caco-2 cells changes the clumping pattern and spatial orientation of bacteria compared with bacterial images without Caco-2 cells (column B). In column C, when bacteria are in media alone, they form clumps and fall to the bottom of the wells attaching to the Caco-2 cell surface. In the presence of PEG 3.35, bacteria are again seen clumped, but they remain suspended above the epithelial cell surface at approximately 8 μm. When bacteria are exposed to PEG 15–20 in the presence of Caco-2 cells, they seem more dispersed and more evenly distributed about the well and remain suspended as much as 32 μm above the epithelial cell surface.

PEG 15–20 that parallel the function of intestinal mucus. Because intestinal mucus exerts its effects on both colonizing bacteria and the intestinal epithelium, both of these compartments were examined in some detail. Similar to the reported effects of intestinal mucus on bacterial growth, we did not observe PEGs to have any effect on bacterial growth patterns.[22] However, like intestinal mucins, PEG compounds affected the clumping pattern of *P. aeruginosa* similarly to that observed for *Escherichia coli* exposed to mouse cecal mucus.[20] Mucus-induced bacterial clumping has been shown to be a major mechanism by which intestinal colonization by luminal pathogens is prevented.[20] In fact, mutanized bacterial strains that can resist mucus-induced clumping have been shown to colonize and invade the mouse intestinal epithelium more efficiently than their parent strains.[20]

A major mechanism by which PEG 15–20 was protective in these studies seemed to be by simply distancing *P. aeruginosa* from the intestinal epithelium. A lack of bacterial contact to the epithelial Caco-2 monolayers in the presence of PEG 15–20 was suggested from experiments that showed preservation of the TEER of the Caco-2 monolayers exposed to PA27853. That PEG 15–20 prevented a contact-induced decrease in TEER after apical exposure of Caco-2 cells to *P. aeruginosa* was first suggested by the finding that counts of nonadherent bacteria remaining in the Caco-2 cell free culture media were 10-fold higher in the presence of PEG 15–20, although bacterial adherence assessed by culture of washed cells was not increased, perhaps because of an inability to wash PEG off of the bacterial/PEG/epithelial cell layer. Nonetheless, fluorescent bacterial images of PA27853/EGFP did show that bacteria are significantly distanced away from Caco-2 cell monolayers exposed to PEG 15–20 to a greater degree than that seen with PEG 3.35, raising the possibility that a critical spatial interface exists between pathogen and host beyond which bacterial pathogenicity does not occur. The importance of assessing these spatial differences beyond bacterial culture may be essential to understanding such events. Others have shown that polymers such as PEGs can exert a repellant or distancing effect to approaching variously charged proteins by undergoing a change in their conformational entropy in response to contact onto a biological surface.[23] The anchoring of PEG 15–20 to both Caco-2 and PA27853 cell surfaces could impose molecular constraints on PEG compounds and result in a loss of elasticity or conformational freedom of the polymer.[24] Alternatively, coating of both epithelial and bacterial cells with PEG 15–20 could lead to an alteration in the

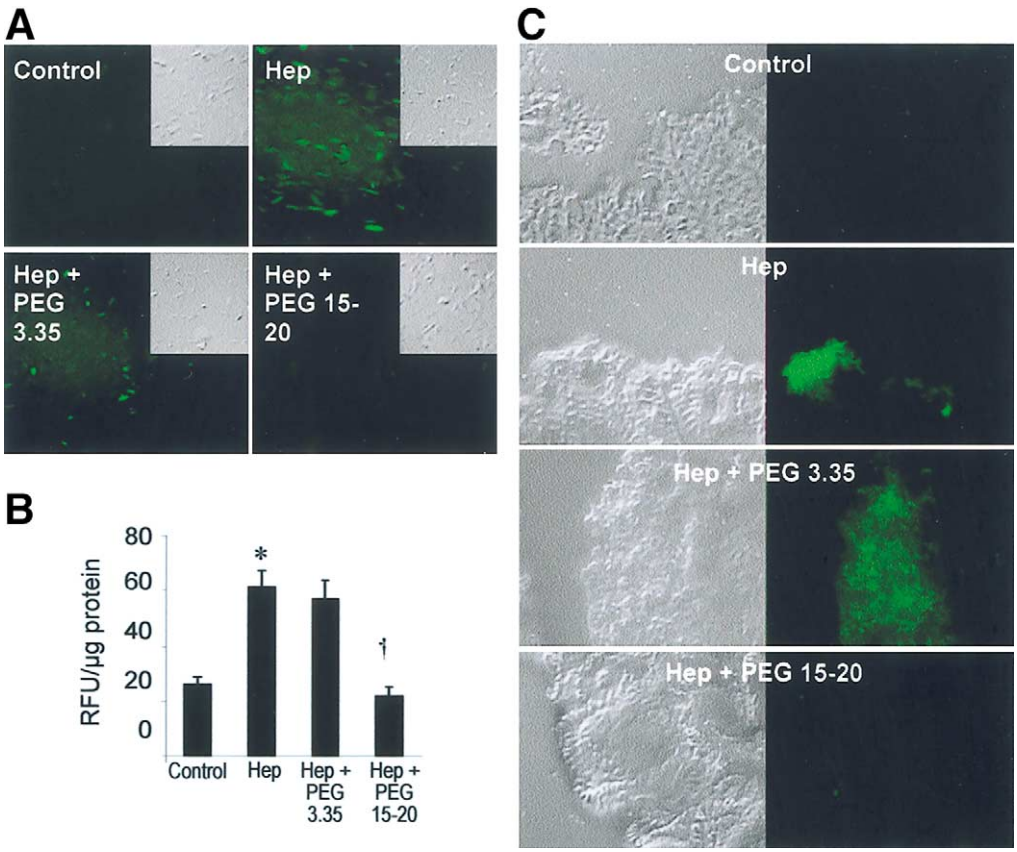

Figure 8. The effect of PEG 15–20 on PA-I expression in vivo. Reporter strain PA27853/PLL–EGFP was introduced into the mouse cecum in the presence and absence of PEG 3.35 and PEG 15–20 after hepatectomy. Mice were killed after 24 hours, and feces and cecal tissues were harvested, processed, and imaged with fluorescence microscopy. Fluorescence was quantified in processed fecal samples (n = 3) by fluorometry of cytosolic fractions of bacterial cells. (*A*) Overlays of images of bacteria harvested from the cecum from the various groups. The presence of PEG 15–20 in the mouse cecum protected against PA-I expression in vivo. (*B*) Quantitation of PA-I expression in feces by fluorometry showing a statistically significant increase (*$P < 0.05$; analysis of variance) after hepatectomy. All data are mean ± SEM. This effect was attenuated in the presence of PEG 15–20 (†$P < 0.05$; analysis of variance). (*C*) Overlays of images of cecal tissues from the various groups. Mice undergoing hepatectomy had visibly fluorescent bacteria on and within the mucosa. Fluorescent bacteria were not seen on or within cecal tissues when mice were pretreated with PEG 15–20. Pretreatment with PEG 3.35 did not prevent PA27853/PLL–EGFP from fluorescing in mice undergoing hepatectomy.

surface electrical charge or hydrophobic characteristics of both biological bodies such that a net repulsive force is generated in response to like charges. Evidence for this notion can be seen in Figure 7, in which PEG solutions alone do not suspend bacteria above the bottom of the culture wells, whereas in the presence of Caco-2 cells, bacteria are seen suspended above the bottom of the culture wells. Collectively, these findings suggest a strong interactive component to the repulsive force of PEGs that seems to be activated when present on Caco-2 cells.

To better understand such forces, both electron microscopy and AFM were used to characterize the surface morphology of bacterial cells in the presence of the 2 PEG molecules. AFM force measurements make it possible to probe the physical properties of the surface of live cells with nanometer resolution[25] (Figure 6). AFM scanning of bacterial cells showed a significant increase in the height diameter of bacterial cells in the presence of PEG 15–20, an effect that appeared as an electron halo around the bacteria by electron microscopy. Although these findings are preliminary, a molecular weight–dependent effect on bacterial cell surface properties seems to be a distinct feature of PEG 15–20. The extent to which these changes direct the behavior and response of bacteria within the intestinal epithelial environment will require more detailed study.

A second major mechanism by which PEG might have been protective in this model was by inhibition of bacterial virulence gene expression. In this study, PEG 15–20 rendered *P. aeruginosa* insensate to both C4-HSL and epithelial cells, as judged by a lack of PA-I expression. Although the PA-I lectin is among the many virulence determinants necessary to initiate epithelial infection, its co-regulation with other virulence genes in response to quorum-sensing signaling molecules makes

it an appropriate "read-out" for virulence gene activation in *P. aeruginosa*. The finding that PEGs can desensitize *P. aeruginosa* to its cognate quorum-sensing molecules is significant, given that quorum sensing controls hundreds of genes that encode for the toxicity of this organism against the host.[17,26] Mutant strains of *P. aeruginosa* that cannot communicate via the quorum-sensing signaling system are seriously impaired in their ability to cause infection in animal models.[27] Yet only PEG 15–20 suppressed PA-I expression in vivo and protected against mortality in mice subjected to catabolic stress. Data from Figure 8 show that there are yet-to-be-described factors released into the intestinal lumen after catabolic stress that activate PA-I expression in *P. aeruginosa*. The extent to which PEG 3.35 and PEG 15–20 can shield *P. aeruginosa* from these inducing molecules and suppress PA-I expression may not be able to be assessed by in vitro experiments alone. For example, PEG 3.35 inhibited PA-I expression in response to C4-HSL but did not protect against Caco-2 cell–induced PA-I expression, unlike PEG 15–20, which protected against both effects. However, PEG 3.35 maintained a significant distancing effect on *P. aeruginosa* from the epithelial surface (Figure 7), although this effect was not to the same degree as that seen with PEG 15–20. Because it is well established that host-cell contact alone is capable of inducing bacterial virulence gene expression,[28,29] the greater ability of PEG 15–20 to distance *P. aeruginosa* from the epithelium, as observed in the in vitro experiments, may have played a significant role in suppressing PA-I expression in vivo, as seen in the experiments in Figure 8. Therefore, although PEG 3.35 did have some effects in vitro, its inability to suppress PA-I expression in vivo may be a critical factor in its inability to protect mice. Finally, major differences in the rate by which PEG compounds are degraded in vivo because of the presence of the commensal flora could explain some of the discordances between in vitro and in vivo experiments.[30] Studies are under way to clarify this issue.

The use of PEG 15–20 as an intestinally applied compound to prevent gut-derived sepsis in immunocompromised individuals harboring opportunistic pathogens may have clinical merit. In this mouse model, PEG 15–20 was protective against fatal gut-derived sepsis due to *P. aeruginosa* when directly introduced into the cecum at the time of infection or when administered as an oral solution after infection. Although other biological effects of PEG 15–20 on the intestinal epithelium—such as its ability to stimulate endogenous mucus or other intestinal defense proteins—may exist, its ability to chemically shield the intestinal epithelium may offer a novel approach to bacterial-mediated disorders of the intestinal tract. In addition, whether PEG 15–20 affects probiotic bacteria will require further study. It will be important to specifically address the role of endogenous mucus on the protective effect of PEG, because many of the observed effects closely mimic those of intestinal mucins. We have administered PEG 15–20 orally to mice for up to 4 weeks without any noticeable change in weight or overall health (data not shown). In addition, PEG 15–20 does not change the Na/H exchange characteristics of cultured Caco-2 cells (data not shown). The lack of an effect of PEG 15–20 on *P. aeruginosa* growth and viability may offer a more ecologically neutral approach to containing virulent opportunistic pathogens in immunocompromised hosts over the current approach of multiple and prolonged antibiotic use.[31,32] Given the completely inert and nontoxic nature of PEGs, the use of PEG compounds to treat and prevent bacterial-mediated disorders of the intestinal epithelium deserves further investigation.

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