Role of the Ceramide-signaling Pathway in Cytokine Responses to P-fimbriated *Escherichia coli*

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Summary

Escherichia coli express fimbriae-associated adhesins through which they attach to mucosal cells and activate a cytokine response. The receptors for *E. coli* P fimbriae are the globoseries of glycosphingolipids; Galα1→4Galβ–containing oligosaccharides bound to ceramide in the outer leaflet of the lipid bilayer. The receptors for type 1 fimbriae are mannosylated glycoproteins rather than glycolipids. This study tested the hypothesis that P-fimbriated *E. coli* elicit a cytokine response through the release of ceramide in the receptor-bearing cell. We used the A498 human kidney cell line, which expressed functional receptors for P and type 1 fimbriae and secreted higher levels of interleukin (IL)-6 when exposed to the fimbriated strains than to isogenic nonfimbriated controls. P-fimbriated *E. coli* caused the release of ceramide and increased the phosphorylation of ceramide to ceramide 1-phosphate. The IL-6 response to P-fimbriated *E. coli* was reduced by inhibitors of serine/threonine kinases but not by other protein kinase inhibitors. In contrast, ceramide levels were not influenced by type 1–fimbriated *E. coli*, and the IL-6 response was insensitive to the serine/threonine kinase inhibitors. These results demonstrate that the ceramide-signaling pathway is activated by P-fimbriated *E. coli*, and that the receptor specificity of the P fimbriae influences this process. We propose that this activation pathway contributes to the cytokine induction by P-fimbriated *E. coli* in epithelial cells.

Uropathogenic *Escherichia coli* attach to uroepithelial cells and elicit a cytokine response in those cells (1). The molecular mechanisms of attachment are well defined, but the transmembrane signaling events that lead to cytokine activation are not known (2).

Attachment is mediated by fimbriae, bacterial cell surface organelles with lectin domains (2). The lectins bind specifically to oligosaccharide epitopes exposed on cell surface glycoproteins or glycolipids. Type 1 fimbriae bind terminal mannose residues, S fimbriae recognize sialic acid determinants, and P fimbriae bind Galα1→4Galβ–containing oligosaccharides, the carbohydrate portion of the globoseries glycosphingolipids (3–6). Fimbriae enhanced the epithelial cell cytokine response to bacteria (7, 8). P-fimbriated *E. coli* elicited a higher IL-6 response than isogenic strains lacking the fimbriae, and isolated P fimbriae with an intact lectin domain triggered an IL-6 response, whereas lectin-deficient fimbriae failed to do so (7). Furthermore, cytokine activation was reduced by treatment that inhibited glycolipid receptor expression by the epithelial cells (9).

These results suggested that fimbriae–glycolipid interactions may trigger transmembrane signaling events involved in the induction of cytokine responses. The oligosaccharide receptor epitopes recognized by P fimbriae are bound to ceramide in the outer leaflet of the lipid bilayer (6, 10). Until recently, glycolipids were not considered to participate in transmembrane signaling, partly because of the lack of a transmembrane domain. Recent studies have demonstrated that ceramide can be released from sphingomyelin (SM)[1] by agonists like TNF-α, IL-1β, or 1α,25-dihydroxyvitamin D_3 (11–13). TNF-α–induced ceramide release has been shown to activate NF-κB (11). Ceramide can also undergo phosphorylation through the action of membrane-bound serine/threonine protein kinases, and ceramide 1-phosphate further activated IL-2 in thymoma cells (12, 14).

The aim of this study was to examine if P-fimbriated *E. coli* activate the ceramide-signaling pathway, and if this can contribute to the induction of an epithelial cytokine response by these bacteria.

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[1]*Abbreviations used in this paper:* αMan, α-methyl-D-mannoside; CAPK, ceramide-activated protein kinase; SM, sphingomyelin; SMase, sphingomyelinase.

Materials and Methods

Materials. BSA, cardiolipin (bovine heart), ceramide (type III), diethylenetriaminepentaacetic acid, genistein, goat anti-rabbit IgG alkaline phosphatase, Hepes, K252a, *n*-octyl-β-D-glucopyranoside, PMA, sphingomyelinase (SMase) (*Staphylococcus aureus*) (E.C. 3.1.4.12), staurosporine, and tyrphostin 51 were from Sigma Chemical Co. (St. Louis, MO). Diacylglycerol kinase (E.C. 2.7.1.107) was from Calbiochem Corp. (San Diego, CA). Smooth LPS (*Salmonella typhimurium* SH4809) was kindly provided by Professor Alf Lindberg (Karolinska Institute, Stockholm, Sweden). Fetal calf serum, L-glutamine, nonessential amino acids, RPMI 1640, sodium pyruvate, and trypsin (E.C.3.4.21.4) were from GIBCO BRL Life Technologies Inc. (Paisley, Scotland, UK). Mouse anti-human mAbs (M8) and recombinant IL-6 were from Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Rabbit polyclonal anti-human IL-6 antibodies were from Genzyme Corp. (Cambridge, MA). [^{32}P]orthophosphate (carrier free) was from ICN Radiochemicals (Irvine, CA). [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International (Amersham, UK). ^{35}S-protein labeling mix (3,000 Ci/mmol) was from DuPont NEN (Boston, MA). HPLC-grade solvents and EDTA (Titriplex III) were from Merck AG (Darmstadt, Germany).

Bacteria. *E. coli* strains with defined P and type 1 fimbrial expression were used. *E. coli* AD110 was a clinical isolate from which the *pap* gene clusters, encoding P fimbriae, has been cloned and sequenced. *E. coli* HB101/pap_{AD110} carried the plasmid pPIL110-35 with a 16-kb EcoRI AD110 *pap* DNA insert in the EcoRI site of pACYC184 (15). *E. coli* PKL4 (AAEC film $_{PC31}$) carried the plasmid pPKL4 with the *fim* DNA sequences from *E. coli* PC31 (16). *E. coli* HB101 was *pap* negative but hybridized with probes for the *fim* DNA sequences. *E. coli* AAEC was *pap* and *fim* negative. Both strains were phenotypically negative for P and type 1 fimbriae. For adhesion testing and cytokine activation, *E. coli* AD110 was cultured on tryptic soy agar plates (TSA; Oxoid Ltd., Basingstoke, Hampshire, UK). *E. coli* HB101/pap_{AD110} and *E. coli* PKL4 were cultured on TSA with tetracycline (10 μg/ml) and ampillicin (100 μg/ml), respectively. Adhesin expression was tested by hemagglutination using human erythrocytes of blood group A_1P_1 and Ap erythrocytes as well as guinea pig erythrocytes (6). P. fimbriae caused mannose-resistant agglutination of human A_1P_1 erythrocytes, which express the globoseries of glycolipids, but not of Ap erythrocytes, which lack these glycolipids (2, 5, 6). Type 1 fimbriae caused mannose-sensitive agglutination of guinea pig erythrocytes that was inhibited by α-methyl-D-mannoside (αMan; 0.1 M) (3).

Epithelial Cell Line. The human epithelial cell line A498 (HTB44, human kidney carcinoma; American Type Culture Collection) was grown in RPMI 1640 supplemented with 5% FCS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, gentamicin (50 μg/ml), and 2 mM L-glutamine. A stock culture was maintained in 75-cm^2 culture flasks (Falcon Labware, Becton Dickinson Ltd., Oxford, UK) at 37°C in a 5% CO_2 atmosphere and split weekly.

Bacterial Adherence Assay. A498 cells were detached by treatment with trypsin (0.25%) for 5 min at room temperature or with 0.5 mM EDTA at 37°C for 15 min, harvested by centrifugation at 150 *g* for 7 min, and washed by repeated cycles of centrifugation and resuspension in 60 mM PBS, pH 7.2. The epithelial cell suspension was mixed with bacteria (final concentration, 10^5 cells and 10^9 bacteria/ml) and incubated for 45 min at 37°C with end-over-end rotation. Unattached bacteria were removed by repeated cycles of centrifugation and resuspension in PBS. The number of bacteria attached to 40 cells was counted by interference contrast microscopy (Nikon Microphot-FX; Nikon, Tokyo, Japan). Adherence is expressed as the mean number of attached bacteria per cell.

Cytokine Responses. The cytokine responses of A498 cells to the difference agonists were analyzed using cells grown to confluency in 24-well cell culture plates (Falcon). The medium was aspirated and replaced by fresh RMPI containing *E. coli* (10^8 bacteria/ml), PMA, (10 ng/ml), or SMase (0.01–1 U/ml). Bacterial multiplication was limited by residual gentamicin.

The effect of protein kinase inhibitors on the cytokine response was tested by adding the inhibitors to the wells 10 min before the agonist. Staurosporine and K252a (inhibitors of serine/threonine kinases) were dissolved in 100% DMSO and diluted in RPMI 1640 medium to a final concentration of 500 nM/well. Genistein and tyrphostin 51 (inhibitors of tyrosine phosphorylation) were dissolved in DMSO and diluted in RPMI 1640 to a final concentration of 10 μM/well.

IL-6 Quantitation. This was by ELISA (17). Briefly, mouse anti-human IL-6 mAbs (M8) (100 μl/well, 5 μg/ml in 0.05 carbonate buffer, pH 9.8) were used for coating. Samples from the A498 cell line or rhIL-6 were diluted in PBS containing 1% BSA (1% BSA–PBS). Bound IL-6 was detected by addition of polyclonal rabbit anti-human IL-6 antibodies followed by goat anti-rabbit IgG alkaline phosphatase. The standard curve was prepared using stock rhIL-6. The activity of each sample was compared with the standard curve and given as picograms IL-6 per milliliter.

Glycosphingolipids. These were extracted from the A498 cell line as described (18, 19). Cells were detached from the culture flasks into medium by mechanical scraping with a rubber policeman, collected by centrifugation at 150 *g* for 10 min, and extracted with water/methanol (1:2) for 1 h at 65°C. After centrifugation at 188 *g* for 10 min, the supernatant was collected, and the cell pellet was reextracted with methanol/chloroform (2:1). The supernatants from each extraction were pooled, and the solvents were removed by evaporation under nitrogen. The lipids were subjected to mild alkaline hydrolysis by 0.2 M KOH in methanol for 2 h at room temperature. After neutralization with acetic acid, the extract was adjusted to chloroform/methanol/water (1:10:9) and desalted on a 3-ml C-18 Bond Elute column (Analytichem International Inc., Harbor City, CA). Desalted lipids were separated by TLC on Kieselgel 60 aluminium-backed HPTLC plates (Merck). The reference Glycolipid was globotetraosylceramide from human erythrocytes (20).

Bacterial Binding to Glycolipids on TLC Plates. Bacterial binding to Glycolipids and Glycolipid extracts was studied by the TLC overlay assay (10, 21). Bacteria were labeled by overnight growth in Luria broth containing 50 μCi of [^{35}S]methionine, harvested by centrifugation at 2,000 *g* for 10 min, and resuspended in PBS to ~10^9 bacteria/ml. The glycolipid extracts were run on TLC plates in chloroform/methanol/water (60:35:8). The plates were treated with 0.2% (wt/vol) polyisobutylmethacrylate in diethyl ether for 1 min and dried at room temperature. To reduce nonspecific binding, the TLC plates were incubated with 2% BSA in PBS for 2 h. Without intermediate drying, the TLC plates were subsequently overlaid with the bacterial suspension and incubated for 2 h. Unbound bacteria were removed by extensive washing with BSA–PBS, and bound bacteria were detected by autoradiography.

Release and Phosphorylation of Ceramide. A498 cells labeled for 72 h with 75 μCi/ml carrier-free $^{32}P_i$ or unlabeled A498 cells were detached with 0.25% of trypsin and washed in PBS. Cell

Table 1. *Fimbrial Expression and Adhesion of E. coli Strains Used in this Study*

E. coli strain	Fimbrial genotype	Hemagglutination		Adhesion to A498 cells (bacteria/cell): −αMan/+αMan
		Human P_1	Guinea pig	
E. coli AD110	*pap*$^+$, *fim*$^+$	MR	—	34/50
E. coli HB101	*pap*$^-$, *fim*$^+$	—	—	0/0
E. coli HB 101/pap AD110	*pap*$^+$, *fim*$^+$	MR	—	4/9
E. coli AAEC	*pap*$^-$, *fim*$^-$	—	—	1/0
E. coli PKL4	*pap*$^-$, *fim*$^+$	MS	MS	21/1

MR, Mannose resistant, not inhibited by αMan; *MS*, mannose sensitive, inhibited by αMan.

pellets were resuspended in RPMI 1640 to a final concentration of 0.3 × 10^6 cells/ml and exposed to bacteria (10^8 CFU/ml) or SMase (1.0 U/ml). Free ceramide and ceramide 1-phosphate were extracted as described (22). Briefly, the reactions were terminated at given time points by lipid extraction of the cell pellets with 1 ml of chloroform/methanol/concentration HCl (100:100:1, vol/vol/vol) and 0.3 ml of balanced salt solution (BSS; 135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, pH 7.2) containing 10 mM EDTA. The organic phase was dried under N_2, and lipids were subjected to mild alkaline hydrolysis by 0.1 M KOH in methanol at 37°C for 1 h. Extracts from unlabeled cells were then incubated with *E. coli* diacylglycerol kinase as described by Preiss et al. (23). Ceramide 1-phosphate from this reaction as well from prelabeled cells was isolated by TLC using chloroform/methanol/acetic acid (65:15:5, vol/vol/vol). Authentic ceramide 1-phosphate (R_F 0.25) was identified and quantitated by image analyzer (BAS 2000; Fuji Film, Tokyo, Japan). The levels of free ceramide from unlabeled cells are expressed as picomoles of ceramide 1-phosphate/10^6 cells. Levels of ceramide 1-phosphate in prelabeled cells are expressed as the percentage of the levels in labeled cells exposed to medium alone.

Assay of SMase Activity. [N-$^{14}CH_3$]choline-labeled milk SM (sp act 56 μCi/mg), synthesized by a demethylation–remethylation procedure (24), was provided by Lena Nyberg (The Swedish Dairy Association, Lund, Sweden). SMase activity was measured according to Gatt (25) with modifications. ^{14}C-SM stored in ethanol was dried under nitrogen and suspended in 0.15 M NaCl containing 3 mM bile salt mixture. Acid SMase activity was measured by adding 25 μl of samples and 0.1 ml of ^{14}C-SM (40,000 dpm) in 375 μl 50 mM Tris-HCl buffer containing 0.15 M NaCl and 3 mM bile salt mixture, pH 5.0. Neutral SMase activity was assayed by adding the samples and ^{14}C-SM to the same buffer supplemented with 2 mM Mg^{2+} and 0.4% Triton X-100, pH 7.5. The incubation was performed at 37°C for 30 min and terminated by addition of 2 ml chloroform/methanol (2:1, vol/vol). After centrifugation and phase partition, an aliquot of the upper phase was taken, and the radioactivity was determined by liquid scintillation counting. The activity was calculated and normalized as picomoles per hour per milligram protein.

Results

Fimbrial Expression and Cell Adhesion of the E. coli Strains. *E. coli* AD110 expressed P fimbriae, but not type 1 fimbriae, under the culture conditions used in this study (Table 1). The attachment to the A498 cells was inhibited by pretreatment of the bacteria with the receptor analogue globotetraosylceramide (data not shown) but not by αMan. The recombinant strain *E. coli* HB101/pap_{AD110} expressed P fimbriae (Table 1). *E. coli* PKL4 expressed type 1 but not P fimbriae, and the attachment to A498 cells was inhibited by αMan. *E. coli* HB101 and *E. coli* AAEC did not agglutinate erythrocytes or attach to epithelial cells. The *E. coli* strains attached in similar numbers to the A498 cell line and to exfoliated human uroepithelial cells (data not shown).

Bacterial Binding to Glycolipids on A498 Cells. Glycolipids were extracted from the A498 cells and separated by TLC. Staining with anisaldehyde showed the presence of glucosylceramides with three to seven sugar residues (not shown). Glycolipids with receptor activity for P fimbriae were detected by overlay with radiolabeled *E. coli* (Fig. 1). *E. coli* HB101/pap_{AD110} recognized glycolipids in the tri-, tetra-, and heptaglucosylceramide regions of the A498 extract and bound to the globotetraosylceramide control, but not to ceramide. The type 1–fimbriated strain *E. coli* PKL4 did not bind to the glycolipids on TLC. Neither did the nonfimbriated control strains *E. coli* HB101 or *E. coli* AAEC.

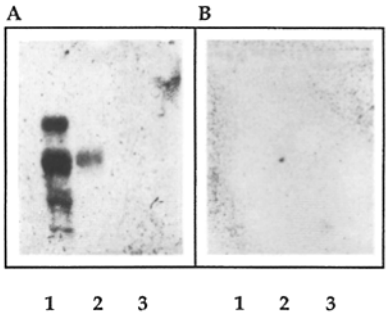

Figure 1. Bacterial binding to glycosphingolipids by TLC overlay. (Lane *1*) Nonacid glycosphingolipid extract from the A498 cell line. (Lane *2*) Globotetraosylceramide. (Lane *3*) Ceramide. TLC overlay with (*A*) radiolabeled P-fimbriated *E. coli* HB101/pap_{AD110}; (*B*) Type 1–fimbriated *E. coli* PKL4.

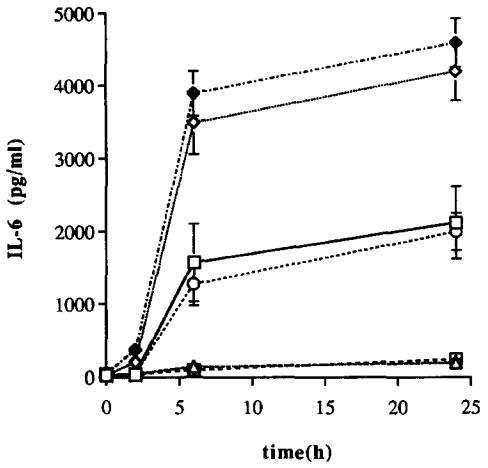

Figure 2. Cytokine response (IL-6) of A498 cells exposed to PMA (◈), *E. coli* PKL4 (type 1 fimbriated) (◇), *E. coli* AD110 (□), and *E. coli* HB101/pap_{AD110} (P fimbriated) (○), *E. coli* HB101 (△), and *E. coli* AAEC (vector controls) (⊞). Means ± SE from three separate experiments.

Attaching Bacteria Elicit a Cytokine Response. The A498 cells were exposed to *E. coli* or PMA. Samples for cytokine measurements were withdrawn at 0, 2, 6, and 24 h. The kinetics of the cytokine responses are shown in Fig. 2. The attaching *E. coli* strains had elicited an IL-6 response above background by 2 h, and the levels remained elevated at 6 and 24 h. The P- and type 1–fimbriated strains and PMA elicited the highest levels of IL-6. The nonfimbriated strains *E. coli* HB101 and *E. coli* AAEC were poor activators of IL-6.

SMase Elicits an IL-6 Response in the A498 Cells. The A498 cells were exposed to SMase (1.0–0.01 U/ml) and samples for IL-6 quantitation were withdrawn at 0, 2, 6, and 24 h. SMase induced a dose-dependent IL-6 response that was detectable above background by 2 h (1.0 U/ml, Table 2) and remained elevated after 6 and 24 h. At 0.01 U/ml, a response was detected only after 24 h. Cells exposed to SMase (1.0 U/ml) or *E. coli* AD110 (10^8 CFU/ml) secreted similar levels of IL-6 secretion. Costimulation of the cells with both agonists had an additive effect on the IL-6 response (Table 2).

Table 2. *IL-6 Response of A498 Cells to E. coli AD110 and SMase*

	IL-6			
Stimulant	0 h	2 h	6 h	24 h
		(pg/ml)		
Control	194	161	320	566
SMase (U/ml)				
1.0	191	230	2,588	3,053
0.1	117	204	377	4,002
0.01	123	127	272	2,801
E. coli AD 110				
(10^8 CFU/ml)	186	966	3,201	3,469
E. coli AD 110				
(10^8 CFU/ml) +				
SMase (U/ml)				
1.0	139	1,867	4,927	5,876
0.1	116	1,178	3,900	3,930
0.01	149	950	2,608	3,523

Release of Ceramide in Cells Exposed to SMase or E. coli. The A498 cells were exposed to medium, SMase (1.0 U/ml), P-fimbriated *E. coli* type 1–fimbriated *E. coli*, or nonfimbriated *E. coli* strains. Ceramide was extracted from cells harvested after 5, 10, 20, and 30 min, and was phosphorylated in vitro using the diacylglycerol kinase assay. The product was separated by TLC, and ceramide 1-phosphate was quantitated by image analyzer. There was a rapid increase in the levels of free ceramide in cells exposed to SMase (151 ± 35% at 20 min) and P-fimbriated *E. coli* (174 ± 22% for *E. coli* AD110 and 125 ± 18% for *E. coli* HB101/

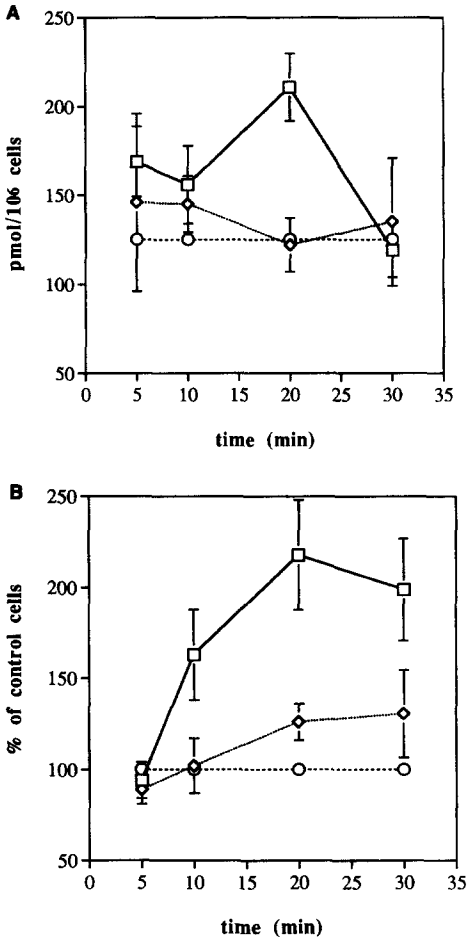

Figure 3. (*A*) Kinetics of ceramide release in A498 cells exposed to *E. coli* AD110 (P fimbriated; □), *E. coli* HB101 (nonfimbriated; ◇), and medium (○). Means ± SE from four experiments. (*B*) Kinetics of ceramide phosphorylation in A498 cells exposed to *E. coli* AD110 (P fimbriated; □), *E. coli* HB101 (nonfimbriated; ◇), or medium (○). Mean ± SE represent duplicate determinations from three experiments.

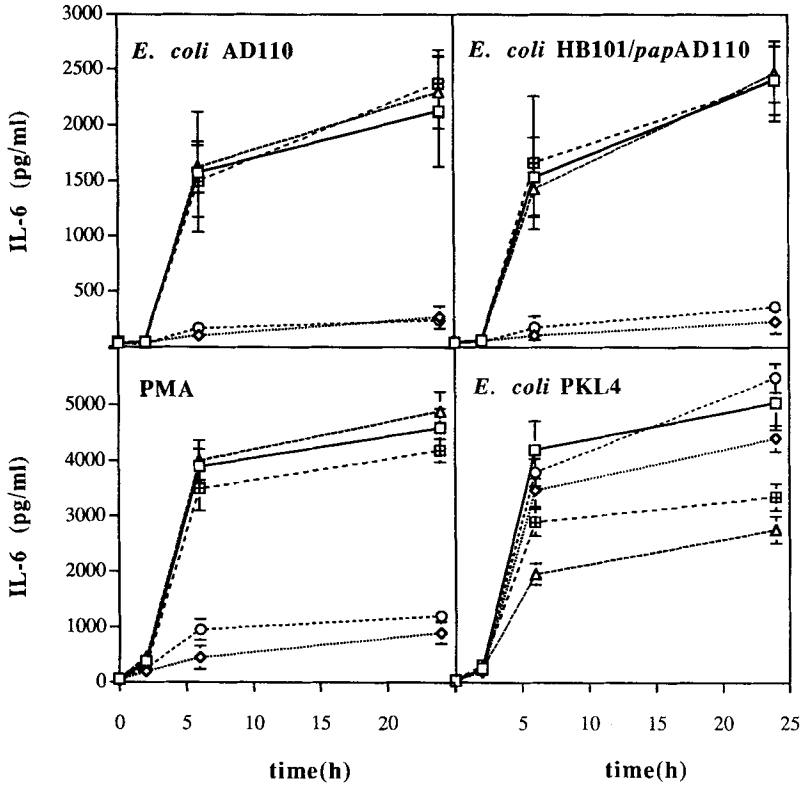

Figure 4. Effect of protein kinase inhibitors on the IL-6 response of A498 cells to *E. coli* AD110 and *E. coli* HB101/pap_{AD110} (P fimbriated), PMA, and *E. coli* PKL4 (type 1 fimbriated). Agonist (□), +staurosporine (◇), +K252a (○), +genistein (△), and +tyrphostin 51 (⊞). Means ± SE from four experiments.

pap_{AD110} at 20 min) (Fig. 3 *A*). The type 1–fimbriated strain, and the nonfimbriated *E. coli* controls, stimulated lower levels of ceramide than the fimbriated strains (89 ± 32% for *E. coli* PKL4, 104 ± 13% for *E. coli* AAEC, and 101 ± 15% for *E. coli* HB101 at 20 min).

Phosphorylation of Ceramide in Cells Exposed to SMase or E. coli. The in vivo phosphorylation of ceramide was studied in cells prelabeled with $^{32}P_i$ for 72 h, and then exposed to medium, SMase (1 U/ml), P-fimbriated *E. coli*, type 1–fimbriated *E. coli*, or nonfimbriated *E. coli*. Cells were harvested at 5, 10, 20, and 30 min, ceramide was extracted, and the amount of ceramide 1-phosphate was determined (Fig. 3 *B*). The control cells had low levels of ceramide phosphorylation. Increased levels were observed in cells exposed to SMase and P-fimbriated *E. coli* for 20 min (223 ± 21% for SMase, 218 ± 30% for *E. coli* AD110, 176 ± 29% for *E. coli* HB101/pap_{AD110}). SMase, the wild-type strain AD110, and the P-fimbriated recombinant strain simulated higher ceramide 1-phosphate levels than the type 1–fimbriated strain and the nonfimbriated strains (133 ± 18% for *E. coli* PKL4, 139 ± 18% for *E. coli* AAEC, and 126 ± 10% for *E. coli* HB101 at 20 min).

Effects of Protein Kinase Inhibitors on Cytokine Responses. Phosphorylation of ceramide has been shown to involve serine/threonine protein kinases. The cytokine response to *E. coli* and PMA was analyzed in cells pretreated for 10 min with staurosporine or K252a (inhibitors of serine/threonine–specific protein kinases) and genistein or tyrphostin 51 (inhibitors of tyrosine-specific protein kinases). The kinetics of the IL-6 response in the presence and absence of the inhibitors is shown in Fig. 4. PMA-induced IL-6 responses were inhibited by staurosporine and K252a, but not by genistein or tyrphostin 51. The IL-6 response elicited by the P-fimbriated strains *E. coli* AD110 and *E. coli* HB101/pap_{AD110} were inhibited by staurosporine and K252a, whereas genistein and tyrphostin 51 had no effect. In contrast, the IL-6 response induced by the type 1–fimbriated strain *E. coli* PKL4 was decreased by genistein and tyrphostin 51 but was not affected by staurosporine or K252a.

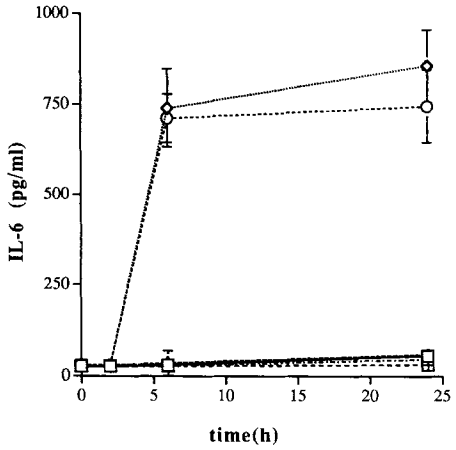

Figure 5. IL-6 response of A498 cells to increasing concentrations of smooth LPS from *S. typhimurium*. 0.1 μg/ml (◈), 1.0 μg/ml (⊞), 10 μg/ml (△), 0.1 mg/ml (○), 1.0 mg/ml (◇), or medium (□). Means ± SE from these experiments.

SMase Production by E. coli and A498 Cells. The SMase activity of the *E. coli* strains was analyzed in culture medium after filtration to remove bacterial cells. Low levels of acid SMase were detected in the bacteria before and after contact with the A498 cells. The SMase activity of the A498 cells was analyzed before and after exposure of the cells to *E. coli*. Both acid and neutral SMases were detected. A moderate increase in endogenous neutral SMase activity occurred after exposure to the bacteria.

LPS Is a Poor Activator of Epithelial Cell IL-6 Responses. The A498 cells were exposed to smooth LPS from *S. typhimurium* (0.1, 1.0, 10, and 100 μg/ml). Samples for IL-6 quantitation were withdrawn after 0, 2, 6, and 24 h (Fig. 5). The cells were negative for expression of surface CD-14 as determined by flow cytometry. Addition of normal human serum did not enhance the IL-6 response of the A498 cells to LPS.

Discussion

Ceramide has recently become recognized as a second messenger in the SM signal transduction pathway. It is released from SM after the action of SMase, an SM-specific form of phospholipase C (26). In cells, ceramide may influence growth and differentiation, regulate protein secretion, induce DNA fragmentation, and apoptosis, and enhance the synthesis and secretion of cytokines (14, 27–32). The molecular mechanisms that control these diverse actions are not yet understood. More is known about the extracellular agonists that cause the release of ceramide. The hydrolysis of SM occurs rapidly upon exposure of the cells to exogenous SMase or to agonists that activate endogenous SMases. Such agonists include TNF-α, IL-1β, IFN-γ, 1α,25-dihydroxyvitamin D_3, and nerve growth factor (12–14, 33–36). The results of this study add P-fimbriated *E. coli* bacteria to the list of agonists that release ceramide and suggest that this signal transduction pathway contributes to the bacterially induced cytokine response in epithelial cells.

Two approaches were used to study *E. coli* activation of the ceramide pathway in the A498 cells. The release of ceramide was studied in unlabeled cells and was quantitated by in vitro phosphorylation with diacylglycerol kinase (23). With this assay, P-fimbriated *E. coli* and SMase were shown to stimulate the A498 cells to release ceramide. The phosphorylation of ceramide to ceramide 1-phosphate was quantitated in cells prelabeled with $^{32}P_i$ and then exposed to bacteria or SMase (22). Extracted ceramide 1-phosphate was quantitiated after separation by TLC. With this assay, P-fimbriated *E. coli* and SMase were shown to induce similar increases in ceramide 1-phosphate levels. These methods were previously used to study the release and phosphorylation of ceramide in EL4 and HL-60 cells (14, 22). The increases in free ceramide and in ceramide 1-phosphate observed in this study were of the same magnitude as the responses to other agonists in previous studies. The kinetics of the responses were also similar. This argues in favor of the bacteria as direct activators of the ceramide pathway and against a two-step process where the bacteria first activate a mediator that, in turn, causes ceramide release. The A498 kidney epithelial cells do not make TNF in response to bacteria, and the IL-1 response is intracellular rather than secreted.

Several intracellular targets for ceramide have been described. The ceramide-activated protein kinase (CAPK) belongs to the serine/threonine protein kinases (14). Other targets include a serine/threonine–specific protein phosphatase and an isoform of protein kinase C (37, 38). In this study, we examined the effects of serine/threonine protein kinase inhibitors on the cytokine response of the A498 cells using PMA, a known activator of these kinases, as a positive control. Staurosporine and K252a markedly reduced the IL-6 response to PMA and to P-fimbriated *E. coli*, suggesting that serine/threonine kinases are involved in the P fimbriae–induced cytokine response. In contrast, tyrosine kinase inhibitors (genistein and tyrphostin 51) had no effect on the IL-6 response.

There were interesting differences in the sensitivity to protein kinase inhibitors between P- and type 1–fimbriated *E. coli*. The P- and type 1–fimbriated *E. coli* strains attached avidly to A498 cells and induced higher IL-6 levels than isogenic, nonfimbriated strains. Whereas the response to the P-fimbriated *E. coli* was blocked by inhibitors of serine/threonine protein kinases, the IL-6 response to type 1–fimbriated *E. coli* was insensitive to these drugs. This suggested that the receptor specificity of the fimbriate and the nature of the cell surface receptor influenced the transmembrane signaling pathway leading to cytokine activation. The P-fimbriated *E. coli* strains induced the release and phosphorylation of ceramide, whereas type 1–fimbriated *E. coli* activated IL-6 production through other as yet unidentified signaling pathways. Type 1 fimbriae bind secreted or cell-bound glycoproteins carrying terminal mannose residues (3). There is no evidence that type 1 fimbriae bind ceramide-containing receptors or that mannose residues recognized by the type 1 fimbrial lectin occur on glycolipids. The P-fimbriated *E. coli*, on the other hand, recognize the Galα1→4Galβ–containing oligosaccharides bound to ceramide in the outer leaflet of the lipid bilayer (2, 5, 6). One might speculate that the P-fimbriated *E. coli* may induce the release of ceramide from the receptor glycolipid rather than from SM. P fimbriae and type 1 fimbriae may also differ in the ability to cause the release of ceramide from SM.

Exogenous SMase was shown to be a potent activator of IL-6 production and to release ceramide in the A498 cells. SMases are produced by a variety of bacterial species. The *S. aureus* SMase used in this study was previously shown to cleave SM and activate the release of ceramide in different cell types (11, 22). Bacterial SMase production could therefore be a mechanism of ceramide release by the bacteria. The *E. coli* strains used in this study had low acid and no detectable neutral SMase activity. Bacterial SMase secretion or upregulation of cellular SMases therefore did not appear to explain the ceramide release and IL-6 response of the A498 cells. Activation of endogenous SMase leads to ceramide release in different cell types. Acid as well as neutral SMase activity were detected in the A498 cells. A moderate

increase in neutral but not acid SMase activity was observed after exposure of the cells to *E. coli*. The possible differences in activation of endogenous SMases related to fimbrial expression need to be explored.

Ceramide and LPS were recently shown to have sufficient structural homology that LPS could replace ceramide as an activator of serine/threonine protein kinases like CAPK (39). The *E. coli* strains used in this study might thus activate cytokine production through a direct effect of LPS on CAPK. Purified LPS was, however, a poor activator of epithelial cell cytokine responses in this and in earlier studies, and the A498 cells lacked surface CD-14 (7, 40, 41). The LPS-induced ceramide phosphorylation was shown to be CD-14 dependent (39).

Furthermore, if LPS were the principal activator of ceramide 1-phosphate production and IL-6 responses, the P- and type 1–fimbriated *E. coli* would be expected to deliver LPS to the surface with similar efficiency. The difference in ceramide release and sensitivity to serine/threonine kinase inhibitors between the P- and type 1–fimbriated strains suggested that P fimbriate contributed in an LPS-independent manner to the response or presented LPS differently to the cells. Earlier studies have suggested that P fimbriae carry LPS at the tip, as an integral part of the G adhesin complex, adjacent to the receptor-binding domain (42). This would provide the basis for a dual signal, through the glycolipid receptor and through LPS. Further studies are required to resolve these questions.

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