Expression of and Cytokine Activation by *Escherichia coli* Curli Fibers in Human Sepsis

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Curli organelles are expressed by commensal *Escherichia coli* K12 and by *Salmonella typhimurium* at temperatures <37°C, which bind serum proteins and activate the contact-phase system in vitro. This study demonstrates, by means of an anti-CsgA (curli major subunit) antibody, that a significant fraction of *E. coli* isolates (24 of 46) from human blood cultures produce curli at 37°C in vitro. Serum samples from 12 convalescent patients with sepsis, but not serum from healthy controls, contained antibodies against CsgA (*n* = 12). This study further demonstrates that a curli-expressing *E. coli* strain and a noncurliated mutant secreting soluble CsgA induce significantly (*P* < .05) higher levels of proinflammatory cytokines (tumor necrosis factor–α, interleukin [IL]–6, and IL–8) in human macrophages differentiated from THP-1 cells. These data, therefore, provide direct evidence that curli are expressed in vivo in human sepsis and suggest a possible role for curli and CsgA in the induction of proinflammatory cytokines during *E. coli* sepsis.

"Septic shock" is defined as a systemic response to infection [1], characterized as an ill-regulated host inflammatory reaction. Mediator systems, such as the complement system [2] and the contact activation system [3, 4], are thought to play a key role in sepsis, via generation of small peptides that can cause an increased vasopermeability and vasodilation. Attention has also been directed to the role of proinflammatory cytokines in septic shock. Relatively small amounts of these cytokines, produced locally in tissue, benefit the host by activating antimicrobial pathways and stimulating tissue repair. However, a massive, uncontrolled release of cytokines and the resultant mediator cascade signal the onset of tissue injury and lethal shock. In this context, regulation of the local inflammatory response, particularly by the proinflammatory cytokines interleukin (IL)–1β and tumor necrosis factor (TNF)–α [5], in response to bacterial invasion is important.

Although much is known about the role of lipopolysaccharides (LPSs) in septic shock [5, 6], little is known about the role of other bacterial components. Several clinical studies have shown that no significant survival advantage results from blockage of LPSs in patients with sepsis [7–9], suggesting that other components of gram-negative bacteria may play a role in the induction and pathology of septic shock.

Curli are a novel class of bacterial surface structures, expressed in both *Escherichia coli* and *Salmonella*, that are characterized by their ability to bind serum protein fibronectin [10, 11]. Colonies of curli-expressing strains typically stain positive for Congo red, allowing for an initial rapid screen of curli production [12, 13]. Considerable work has been done to elucidate the nature and biological role of curli in both *E. coli* and *Salmonella* infection. It is now well appreciated that curli are assembled, following a unique pathway, by bacterial secretion of soluble CsgA, the major subunit protein of curli [14], triggered by a surface-located nucleator protein, CsgB, and are polymerized into curli fibers at the bacterial cell surface [15]. Curli are able to bind different human proteins of the fibrinolytic and coagulation cascades [16, 17] and can also interact with major histocompatibility complex class I molecules [18]. More recently, it has been reported that curli are capable of activating the contact-phase system in human plasma in vitro, allowing an anticoagulation effect and generation of the cellular mediator bradykinin [19]. However, because expression of curli in vitro is temperature regulated and curli fibers normally are not expressed in *E. coli* K12 and in *Salmonella typhimurium* at 37°C, it could be argued that these bacterial fibers do not play any role in the infection of warm-blooded animals and humans.

In the present investigation, *E. coli* isolates from blood cultures of patients with sepsis were analyzed for curli expression at 37°C by use of a specific anti-CsgA antibody. By using immunoblotting assays, we obtained serum samples from patients with sepsis and from healthy controls and analyzed them for antibodies directed against CsgA, the curli major subunit protein. To address a possible role of curli in sepsis, we measured...
the induction of the proinflammatory cytokines TNF-α, IL-6, and IL-8 in human macrophages after exposure to a curli-expressing E. coli K12 strain and its corresponding csgA and/or csgB mutants.

Materials and Methods

Patients. Twelve patients with E. coli bacteremia (6 men and 6 women, with an age range of 22–88 years) were treated at Karolinska Hospital, Stockholm, Sweden. All patients presented with fever >39°C and had general signs of sepsis. The portal of entry was the urinary tract in 7 patients, all of whom had positive urine cultures. Five patients were treated with combinations of gentamicin, 4 patients with a single therapy of trimethoprim-sulfamethoxazole, 1 patient with cefuroxime, and 1 patient with gentamicin, and 1 patient did not receive any antimicrobial therapy. Two patients died after ~1 month, but in neither of these cases could death be referred to the E. coli sepsis.

Patient sera. Convalescent serum samples were obtained from 12 patients with E. coli bacteremia, on average, 16 days (range, 9–29 days) after admission to the hospital. Serum samples from 12 healthy volunteers were used as controls.

Bacterial strains. Forty-six E. coli strains (no. 1–no. 50) from blood cultures of septicemic patients, including the 12 patients mentioned earlier, were collected at Karolinska Hospital. These included MC4100, a curli-expressing strain that is a derivative of E. coli K12 [20], and MHR222, MHR204, and MHR261, isogenic curli-deficient mutant strains generated by transposon insertional mutagenesis of the csgA and csgB genes in MC4100 [13]. Bacteria were grown on colonization factor agent (CFA) agar [21] at 28°C for 48 h or at 37°C for 24 h. Media supplemented with Congo red (40 μg/mL) and Coomassie brilliant blue (20 μg/mL) were used for curli indicator plates to judge colony morphology and color (figure 1). Scores were given as (1) – (negative result); (2) +, +, +, or + + + (Congo red staining positive with light to dark red color); or (3) +++++ (score of +++++ plus an rdar morphotype [22]. Bacterial cells collected from the agar plate were used for immunoblotting, fibronectin-binding, and cytokine induction experiments in macrophages.

Polymerase chain reaction (PCR). Oligonucleotide primers used to amplify a portion of the csgA gene were 5′-GGCCGGAAA-TGGTTTCAGATGTTG-3′ and 5′-CGTATTCATAAGCTTCTC-CCGA-3′. PCR was done in a 100-μL reaction containing 50 pmol of each primer and 250 μM of each dNTP in Taq reaction buffer (10 mM Tris-HCl at pH 8.3, 1.5 mM MgCl2, and 50 mM KCl). Crude chromosomal DNA isolated from E. coli cells was used as a template. Amplification was done on an automatic thermocycler (Perkin-Elmer, Santa Clara, CA). Each reaction was electrophoresed through a 3% agarose gel to verify production of the appropriate fragments.

Immunoblot. Bacteria were grown on CFA agar plates at 28°C for 48 h or at 37°C for 24 h. Bacterial cells were treated with 90% formic acid prior to electrophoresis and were resuspended in protein sample buffer. The constituent proteins were separated on a 15% SDS polyacrylamide slab gel [23]. Immunoblot experiments were performed essentially as described elsewhere [24]: The proteins were transferred onto a nitrocellulose membrane with a pore size of 0.45 μm (Immobilon P; Millipore, Sundbyberg, Sweden) at 100 V for 60 min. The membrane was soaked in 5% nonfat milk in Tris-buffered saline (TBS; 10 mM Tris-HCl and 150 mM NaCl at pH 7.5) for at least 30 min. For the analysis of clinical serum samples, the membrane was cut into 5-mm-wide slices, and each slice was put into one of the small individual tanks of an AutoBLOT 36 (GeneLabs Diagnostics, Singapore). The membrane slices were incubated with septic sera or nonseptic sera for 60 min at room temperature with 1 : 400 dilution in TBS containing 0.5% bovine serum albumin (BSA). Alkaline phosphatase–conjugated goat antihuman IgG (DakoCatts, Stockholm) at 1 : 4000 dilution was used as the second antibody. Colored alkaline phosphatase products on the nitrocellulose sheet were developed at room temperature with nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate in alkaline phosphate buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 5 mM MgCl2 at pH 9.5). For the analysis of clinical E. coli isolates, the membranes were blocked and incubated with a 1 : 4000 dilution of anti-CsgA antibody ZB-αA; a secondary goat antibody against rabbit IgG conjugated with horseradish peroxidase was used for detection, according to the protocol of the manufacturer (Roche, Bromma, Sweden).

Electron microscopy. Electron microscopy (EM) was performed on an EM 109 electron microscope (Phillips Zeiss, Eindhoven, The Netherlands) at 60–80 kV with copper or nickel grids (200- or 300-mesh, respectively) coated with thin films of 2% Formvar (Sigma, St. Louis). After a 24-h incubation at 37°C, the bacterial colonies were overlaid with PBS (pH 7.4), and cell suspension was allowed to sediment for 2 min on a grid. After washing with PBS and then distilled water, the specimen was negatively stained with 0.2% uranyl acetate and was air-dried before the EM study was performed. For the immunoelectron microscopy, specimens were blocked with 1% BSA/PBS and then incubated with ZB-αA (1 : 200 dilution in 1% BSA/PBS) for 60 min at 37°C. After washing with PBS, the specimen was incubated with gold particle–labeled protein A (10 nm colloidal gold particles) for 30 min at 37°C, rinsed with PBS and then distilled water, and negatively stained.

Assay of fibronectin binding. Quantitation of nonradiolabeled fibronectin binding to E. coli bacteria was modified according to the method described by Flock et al. [25]. Bacteria were grown on CFA agar plates at 28°C for 48 h or at 37°C for 24 h. Microtiter wells (Dynatech Laboratories, Chantilly, VA) were coated with 100 μL of bovine fibronectin (Sigma), ranging from 0.08 to 10 μg/mL, at 4°C overnight and at room temperature for an additional 2 h. After blocking with 2% BSA in PBS (PBS with 0.05% Tween 20) for 2 h at room temperature, 200 μL of PBS with 4 × 105 cells/mL bacteria (OD600 0.94–0.95) was added to the wells and incubated at room temperature for 3 h. The wells were washed, and the amount of 100× light absorbance of the adherent bacteria was determined by use of a microplate reader (A600). Each value was obtained from the mean ± SD of 4 independent assays.

The THP-1 cell line and its differentiation into macrophages. The human monocytic cell line THP-1, originally derived from a child with acute monocytic leukemia [26], was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 medium (Sigma) containing 10% heat-inactivated myocyte (GibcoBRL, Paisley, UK), 2 mM L-glutamine (Sigma), 10 mM HEPES (GibcoBRL), and 50 μM 2-mer-
Figure 1.  

A. Congo red-staining phenotype of representative sepsis *Escherichia coli* isolates at 37°C in vitro. Bacteria were grown at 37°C for 24 h on colonization factor agent (CFA) agar plates containing Congo red dye. Levels of Congo red staining are indicated as + to ++++ (Materials and Methods) on the left, and numbers of representative sepsis *E. coli* isolates are indicated on the right of the bacterial colonies.

B. Western immunoblots of curli fibers and CsgA protein produced by sepsis *E. coli* isolates at 37°C in vitro. Sepsis *E. coli* isolates were grown on CFA agar plates at 37°C for 24 h, and MC4100 (a), MHR261 (b), and MHR204 (c) were grown at 28°C for 48 h. Bacterial cells were treated with 90% formic acid before electrophoresis to depolymerize the curli fibers. Proteins of bacterial lysate were separated with 15% SDS-PAGE, transferred onto blotting membrane, and analyzed with the CsgA-specific antibody ZB-aA. The locations of slot and molecular markers (in kDa) are indicated on the right. P, CsgA polymers (curli fibers); D, CsgA dimers; M, CsgA monomers.
captoethanol. The cells were grown at 37°C in a humidified 5% CO₂, 95% air atmosphere for 48 h in a cell culture flask (Nunc, Roskilde, Denmark). The cells were thereafter differentiated with 10 nM phorbol myristic acid (Sigma) at 5 × 10⁵ cells/mL for another 48 h in a Teflon-coated bottle. The expression of surface antigens (CD14, CD45, and HLA-DR) was analyzed with flow cytometry, showing that >96% of the cells had turned into macrophages.

Stimulation of macrophages. The differentiated THP-1 cells were seeded at 10⁶ cells in 1-mL volumes of the complete medium in 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA). The cultures were prepared in duplicate and stimulated with either E. coli MC4100 or the mutants MHR222, MHR204, and MHR263 at the concentration of 10⁷ bacteria/mL (bacterium-to-cell ratio, 10 : 1). Supernatants were removed from the wells at 0 (data not shown), 1 (data not shown), 2, 3, 4, 5, 6, and 7 h (data not shown) poststimulation.

ELISA for cytokine production. Supernatant samples of cell cultures were stored at −20°C before the assays. The amounts of TNF-α, IL-6, and IL-8 in cell culture supernatants were quantified by use of ELISA (BioSite AB, Stockholm) according to the manufacturer. The detection limits were 25 pg/mL for TNF-α, 6.25 pg/mL for IL-6, and 62.5 pg/mL for IL-8, respectively.

Statistical analysis. Comparison of apical versus basal cytokine activity was done by use of the paired Student’s t test. Comparison between groups was done by multiple analysis of variance with the post hoc Tukey-Kramer multiple comparison test. All data are reported in absolute values, where indicated, ± SD. Statistical significance was considered to be achieved at P < .05.

Results

Pathogenic E. coli isolates from human blood cultures express curli at 37°C in vitro. E. coli K12 expresses curli only when bacteria are growing at a low temperature [12, 13], making it doubtful that these highly interactive fibers could play a role in human disease. To further address this question, we analyzed 46 clinical E. coli isolates from blood cultures of patients with sepsis by use of a nonradio labeled assay of fibronectin-binding and an immunoblotting assay with a CsgA-specific antibody, ZB-αA [15].

Among the 46 E. coli isolates, 31 colonies were stained with Congo red (see Materials and Methods) when grown at 28°C, and 24 colonies stained positive at 37°C, ranging from + to ++++ (figure 1A). Colonies of E. coli isolates 1, 10, 30, 36, and 36 were stained only when grown at 28°C (+ to ++++), but not at 37°C (−), thereby showing thermoregulation, as in E. coli K12. Colonies from 2 isolates, 7 and 31, were more heavily stained with Congo red when grown at 37°C than at 28°C, indicating that more curli fibers were produced by these isolates at the higher temperature, which suggests a reversed thermoregulation for these 2 isolates.

Curlated E. coli typically bind soluble fibronectin [10, 12]. Twenty-two of the 46 E. coli isolates, including 4 Congo red-binding negative isolates grown at 28°C or 37°C, were therefore tested for fibronectin binding by use of a nonradio labeled assay (table 1). The colonies of isolates that stained differently with Congo red (from − to ++++) bound fibronectin, ranging from 5 to 103 (98 for MC4100 and 12 for MHR204 when grown at 28°C), meaning that most of the isolates that stained positive with Congo red also were capable of binding human soluble fibronectin.

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NOTE. −/−, negative for both CRS and WB assays; + to ++++, CRS positive with light to dark red color; ++++/+, CRS with +++ plus an edar morphotype.

a Each FN-binding value is the amount of 100× light absorbance (mean ± SD).
In addition, all 46 E. coli isolates were analyzed by immunoblotting with the specific anticurli subunit CsgA antibody, ZB-αA (figure 1B). Bacterial cells were treated with 90% formic acid before electrophoresis to depolymerize curli fibers into CsgA monomers. Specific signals of CsgA monomers, CsgA dimers, and CsgA polymers (curli fibers) were detected from the isolates that also stained positive on Congo red plate and bound fibronectin at 28°C or 37°C. From these data, we conclude that a significant fraction (24 of 46) of human E. coli sepsis isolates are capable of producing curli at 37°C in vitro. We also used PCR to test 4 sepsis isolates unable to produce curli in vitro at either 37°C or 28°C for the presence of the csgA gene. As shown in figure 2, all 4 sepsis strains yielded a PCR fragment of the same size as in MC4100. No such DNA fragment was amplified in E. coli MHR206 [13], because disruption (csgD1 : : Tn103) of the csgD gene completely abolishes transcription of the csgBA operon. Hence, likely most, if not all, of the E. coli sepsis isolates contain the genetic information for curli expression.

Different expressing levels of curli fibers among sepsis E. coli isolates. The level of CsgA polypeptide differed among isolates according to the results of the Western blot assay (figure 1B) and, in general, corresponded to the different degree of Congo red staining and fibronectin binding (table 1). Some of the sepsis E. coli isolates, including 22 and 32, were examined by immunoelectron microscopy. As exemplified in figure 3, E. coli 22 (panel A) expressed considerably fewer curli fibers (+) at 37°C than did E. coli 32 (panel B). Thus, the different levels of Congo red staining and fibronectin binding seen at 37°C probably reflect different expression levels of curli fibers among different isolates.

Septic sera, but not sera from healthy controls, contain antibodies against the curli major subunit CsgA protein. As shown in figure 4, a protein band (lanes +, MC4100 lysate) corresponding to the major subunit protein CsgA reacted specifically with anti-CsgA antibody, ZB-αA (lane A+, positive control), and all the septic sera of convalescence (lanes 17+ to 46+) but not with the sera from healthy controls (figure 4H, lanes 1H–12H). Membranes transferred with proteins of MHR204 lysate (lanes –, lacking CsgA) did not react with ZB-αA (lane A–, negative control) or the septic sera (figure 4I), indicating that the septic sera contain antibodies directed against the curli subunit protein CsgA. It is therefore suggested that E. coli associated with sepsis express curli fibers (polymerized CsgA protein) in vivo.

Curli-expressing and noncurliated, but CsgA-secreting, E. coli strains induce higher levels of TNF-α, IL-6, and IL-8 than do CsgA-negative mutants. Figure 5 illustrates the kinetics of cytokine production in human macrophages differentiated from monocytic THP-1 cells. The macrophages were stimulated by infecting them with the curli-expressing wild-type strain MC4100, the noncurliated but CsgA-secreting strain MHR261, the csgA mutant MHR204, and the csgBA double mutant MHR222. All 4 E. coli strains induced a time-dependent increase in TNF-α, IL-6, and IL-8 production in macrophages. Nonstimulated cells produced very low levels of TNF-α and IL-8 and an undetectable level of IL-6. At 3 h after infection, the levels of TNF-α, IL-6, and IL-8 stimulated by MHR222 and MHR204 were significant but were lower than those seen after MC4100 and MHR261 stimulation. However, at 5 and 6 h after stimulation, the levels of all 3 cytokines were significantly higher (P < .05) when cells were induced by MC4100 (except for TNF-α at 5 h) and MHR261 than when cells were induced by MHR222 and MHR204, demonstrating a striking difference depending on the presence or absence of the CsgA protein. The most dramatic difference between the strains was seen for IL-6.

Figure 2. Amplification of csgA gene by polymerase chain reaction in sepsis Escherichia coli isolates. A portion (295 bp) of DNA fragment was amplified from crude chromosomal DNA and was isolated from sepsis E. coli 19, 21, 24, and 25 (grown at 37°C for 24 h) and the wild-type curli-expressing E. coli MC4100, as well as the csgD mutant MHR206 (28°C for 48 h), respectively. The size of DNA markers (Mr) is indicated on the left in base pairs.
Both the curli-producing strain MC4100 and the curli-deficient but CsgA-secreting mutant MHR261 gave rise to a 5-fold higher level of IL-6 at 5 h after infection and a 4-fold higher level at 6 h after infection than those of the noncurliated mutants lacking CsgA. The activity of the cytokines reached a maximal level at 6 h and then decreased rapidly (data not shown), which likely reflects the nutrient consumption by proliferation of bacteria and the decreasing number of viable macrophages.

Dose-dependent induction of TNF-\(\alpha\) by curli-expressing E. coli. It is impossible so far to purify native curli fibers, because all of the protocols applied for curli purification involve a denaturation process with detergent and formic acid. To see if cytokine (TNF-\(\alpha\)) induction is dose dependent, macrophages were infected for 5 h with different amounts (\(10^4\)–\(10^8\) cfu) of the curli-expressing strain MC4100 and the csgBA double mutant MHR222, respectively (figure 6). Both MC4100 and MHR222 induced a dose-dependent increase in TNF-\(\alpha\) production in macrophages. However, the levels of TNF-\(\alpha\) were significantly higher (\(P < .05\)) when macrophages were infected with \(10^6\)–\(10^8\) cfu of MC4100 versus the same concentrations of MHR222, strongly suggesting that curli (CsgA/CsgB) contribute significantly to TNF-\(\alpha\) production by intact bacteria.

A sepsis E. coli isolate expressing curli induces higher TNF-\(\alpha\) production in vitro than does sepsis isolate lacking curli. Two sepsis E. coli isolates with the same serotype (O1K1), 32 (curli positive at 37°C) and 30 (curli negative at 37°C), were compared for their ability to induce TNF-\(\alpha\) production in human macrophages (figure 7). Macrophages were infected with \(10^6\) cfu of sepsis E. coli 32 and 30, respectively. At 5 h after infection, a significantly higher (\(P < .05\)) level of TNF-\(\alpha\) was induced by 32 than by 30. A similar level of TNF-\(\alpha\) was induced by \(10^6\) cfu of MC4100 at 5 h after infection. There was no statistical difference in the cytokine production between 30 and MHR222.

Discussion

E. coli and Salmonella frequently express thin, highly aggregated surface organelles, curli, when grown at temperatures <37°C [10–13]. It has therefore been questioned whether these curli fibers play any role in diseases of warm-blooded animals. In the present study, we demonstrate that >50% (24 of 46) of clinical E. coli isolates from blood cultures of patients with sepsis were capable of producing curli at 37°C in vitro, as indicated by Congo red staining and fibronectin binding and verified by immunoblotting assays (table 1, figure 1).

Congo red binding has been used as an indicator of surface hydrophobicity for certain pathogens [27]. By making use of a Congo red staining assay [12, 13], we were able to screen quickly for in vitro expression of curli fibers among many E. coli isolates at laboratory conditions. We found that 46 clinical E. coli isolates express a variable Congo red-binding pattern when grown at either 28°C or 37°C, giving scores from + to ++++ (see Materials and Methods and figure 1A). In most cases, when the colonies stained with the dye, bacteria were expressing curli fibers, as judged by immunoblotting with specific antibodies to the major curli subunit protein CsgA (figure 1B). However, the degree of staining and morphology of bacterial colonies varied on curli indicator plates when viewed by use of EM, depending on the expression level of curli fibers (figure 2). Colonies from 4 bacteremic isolates (5, 8, 26, and 30) stained slightly (+) with Congo red when grown at 37°C, but no curli signal was detected.
Figure 4. Western blot to probe for antibodies to the curli major subunit CsgA in serum samples from sepsis patients. Curli-expressing wild-type *Escherichia coli* strain MC4100 and *csgBA* double mutant MHR222 were grown on colonization factor agar plates for 48 h at 28°C. Bacterial cells were treated with 90% formic acid before electrophoresis. Proteins of bacterial lysate from MC4100 or MHR222 were transferred onto 2 blotting membranes, which were cut into 5-mm-wide slices. Each membrane slice was put into a small tank in AutoBLOT 36 (GeneLabs Diagnostics, Singapore) and incubated 1:400 with the septic serum or healthy serum for 60 min at room temperature. I, lanes 1: MC4100; lanes 2: MHR222; lane A: probed with ZB-aA; lanes 17–46: analyzed with the convalescent sera from different septic patients. II, lanes 1: MC4100; lanes 2: MHR222; lanes H1–H12: analyzed with sera from healthy controls. Locations of CsgA monomers and molecular weight are indicated on the left in kDa.

by immunoblotting. However, in no case did we detect a CsgA signal by immunoblotting from colonies negative for Congo red staining. Consequently, Congo red staining in general is a quick and efficient assay to screen for curli production in *E. coli* but is not absolutely accurate.

Fibronectin binding is one of the unique features of curli-expressing bacteria of both *E. coli* and *Salmonella* [10, 11]. We have shown in the present study, by using a nonradiolabeled assay, that clinical *E. coli* sepsis isolates bind to soluble fibronectin when they are expressing curli fibers (table 1). Thus, curli-expressing clinical isolates growing either at 28°C or 37°C (judged by immunoblotting assay) gave different values of light absorbance ranging from 11 to 103. Colonies with higher scores on curli indicator plates showed the highest level of fibronectin binding, with a light absorbance similar to that of curli-expressing MC4100. The isolates that were negative by immunoblotting uniformly showed a low light absorbance, similar to that seen for the *csgA* mutant MHR204. A few sepsis *E. coli* isolates—for example, 41 and 46—gave a high score (++++) by means of Congo red staining but showed a relatively low fibronectin-binding activity. Bacteria from such colonies are highly adhesive, forming aggregates at the bottom of the wells. Such aggregates may sometimes cause problems in measuring the light absorbance, which may explain the discrepancies found in the fibronectin-binding assay.

The most direct evidence of curli expression makes use of a CsgA-specific antibody. A significant fraction (24 of 46) of human *E. coli* sepsis isolates were capable of producing curli fibers at 37°C in vitro, and 1 isolate did not express curli at 28°C. Only 7 isolates in the collection expressed curli only at 28°C but not at 37°C, such as *E. coli* K12. We do not know yet whether isolates expressing curli at 37°C contain adaptory mutations in the *csgD* promoter affecting sigma-factor recognition, as has recently been shown for *S. typhimurium* producing curli at 37°C [22]. Curli production is under a highly complex regulation involving not only the transcriptional regulator CsgD [13] but also OmpR, a regulator belonging to a 2-component signal transduction system. Mutations in the *ompR* gene were recently shown to affect curli production in *E. coli*, even though the underlying mechanisms are not clear [28]. In *S. typhimurium*, the expression of curli can be induced at the higher temperature (37°C) by iron limitation [22]. It may therefore be that an even higher percentage of *E. coli* are capable of producing curli at 37°C when growing in the bloodstream, a compartment low in available iron.

*E. coli* is the most common gram-negative organism isolated from patients with sepsis [29]. Recently, it was demonstrated that curli-expressing *E. coli* and *Salmonella* are capable of activating the contact-phase system in human plasma, allowing for an anticoagulation effect and generation of the cellular mediator bradykinin [19]. It has therefore been speculated that curli expression in bacteria may contribute to the coagulation
abnormalities and blood pressure fall seen during sepsis. However, this important study did not reveal whether curli are expressed during sepsis.

In the present study, we analyzed serum samples from 12 convalescent patients with *E. coli* sepsis and serum samples from 12 healthy individuals. We found antibodies to the curli subunit CsgA only in the former group, and even sepsis patients with the isolated *E. coli* strains 19, 21, 24, and 25 did not produce curli fibers in vitro (table 1, figure 1). Together, these data provide evidence that curli fibers are expressed in vivo in human sepsis. It is conceivable that only strains producing very high levels of curli during infection could produce organelles in such quantities that they could affect the coagulation status and generate sufficient levels of bradykinin to induce symptoms.

As an initial phase in the infectious process, most pathogenic bacteria must be able to attach to epithelial surfaces, which is frequently due to the expression of adhesive surface organelles, such as fimbriae that recognize eukaryotic cell surface receptors. Attachment may trigger a signal transduction cascade that results in the upregulation of proinflammatory cytokines [30]. Attached bacteria may also deliver effector proteins into the cytosol or nucleus of host cells or tissue, affecting the transcription of genes for different cytokines if the organism contains a type III secretion system [31]. However, adhesins might also allow host cells to better recognize those patterns on microbes, such as LPSs and other bacterial products that are known to activate innate immune responses [32], an issue that needs to be addressed in light of the results presented here.

We demonstrate here that curli are responsible to a significant degree for the induction of TNF-α, IL-6, and IL-8 when human macrophages are infected with *E. coli* strain MC4100 that is grown on plates but is not expressing type 1 fimbriae or any other known adhesive structure. Strikingly, the noncurliated *E.
coli mutant MHR261, which secretes soluble CsgA, also acts as a potent cytokine inducer, suggesting that it is not curli-mediated attachment to macrophages that is responsible for the massive induction of cytokine, but rather it is the CsgA protein itself, irrespective of whether CsgA is present as a curli-CsgA polymer on the bacterial surface or as a secreted soluble CsgA monomer. To our knowledge, this is the first time that a fimbral subunit monomer is shown to have a role in cytokine induction. At present we do not know whether secreted CsgA acts directly to activate innate immune responses or whether it contains a sufficient amount of bound LPSs to trigger cytokine induction through the LPS pathway, thereby acting as a signaling bullet. Interestingly, a partially purified Salmonella flagellin, Fllic, the main subunit of the flagellar filament, has recently been shown to cause TNF-α induction in human promonocytic cells [33], suggesting that the assembly of flagellin into filaments is not required to achieve a TNF-α response. However, the possibility cannot be ruled out that other bacterial components may be present in the crude flagellar preparation and may contribute to TNF-α induction.

It has been shown elsewhere that type 1 and P-fimbriae-positive E. coli strains stimulate the production of IL-1α, IL-1β, TNF-α, IL-6, and IL-8 in peripheral blood monocytes; IL-6, IL-8, and IL-1α were induced in a bladder epithelial cell line; and only IL-6 and IL-8 were induced in a kidney cell line [34]. Activation of the ceramide-signaling pathway contributes to the cytokine induction by P-fimbriated E. coli in epithelial cells [35]. Subsequently, fimbiae-associated adhesins of S. typhimurium were shown to induce increased levels of mRNA for IL-1β, IL-6, granulocyte-macrophage colony-stimulating factor, chemokines, and melanocyte-growth stimulatory activity in murine macrophages [36]. However, some virulent strains of intracellular bacteria do not induce TNF-α. Hence, Brucella suis releases an inhibitor for the release of TNF-α from E. coli-infected macrophages [37], and Yersinia enterocolitica encodes a suppressor, YopP (YopJ in Y. pseudotuberculosis), which impairs activation of NF-κB and thereby suppresses TNF-α production [38–40]. Thus, bacteria have evolved different means of inducing and manipulating the host cytokine network.

TNF-α is considered a central mediator in septic shock, and it plays an important role in host defense against intracellular pathogens [41, 42]. Together with IL-1, TNF-α is necessary to keep infections localized and prevent the development of sepsis, but once an infection has become systemic, the effects of IL-1 and TNF-α are detrimental, and sepsis ensues. IL-6 is another cytokine recognized to play a role in sepsis that frequently is found in high concentrations in sepsis patients; circulating levels of IL-6 correlate with clinical outcome [43, 44]. A variety of cells, including macrophages and monocytes, produce IL-8, which shows a remarkable specificity for neutrophils [45]. IL-8 is resistant to chemical or proteolytic inactivation, and its biological activity in inflamed tissues may last for several hours [46]. However, the precise role of IL-6 and IL-8 in the pathogenesis of sepsis is still not well established.

Although LPSs are very important mediators of sepsis, other factors must also be involved [47], because LPS-insensitive mice remain vulnerable to gram-negative sepsis [48, 49]. In fact, there is significant evidence that other components of gram-negative bacteria also play an important role in the pathologic changes.
that are associated with infections mediated by these organisms [50]. The case illustrated herein shows that bacterial curli fibers and/or secreted curli subunit monomers affect proinflammatory cytokine induction. It may be beneficial for the septic host if the biological effects of curli and its CsgA subunit could be neutralized by blocking antibodies or by other means, even though the precise role of these effects during infection remains to be determined.

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