Extracellular Release of Reactive Oxygen Species from Human Neutrophils upon Interaction with Escherichia coli Strains Causing Renal Scarring

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Escherichia coli strains that cause acute pyelonephritis possess special combinations of virulence factors. Many of these factors are missing in strains isolated from pyelonephritis patients who develop renal scars. We have therefore suggested that other bacterial characteristics are of importance for the development of the scars and/or that the host defense plays a more significant role than the bacteria in this process (30). Inflammatory cells, especially the professional phagocytes (i.e., neutrophil granulocytes and macrophages), are essential for the elimination of bacteria from the urinary tract (39). To kill invading microbes, the neutrophils are equipped with an arsenal of bactericidal molecules (17), involving both oxygen-independent and oxygen-dependent mechanisms. The oxygen-dependent pathway is characterized by an increased uptake of molecular oxygen, followed by superoxide anion (O2−) and hydrogen peroxide (H2O2) formation (2, 8, 22). Together with myeloperoxidase and a halide, H2O2 constitutes a powerful antimicrobial system. A fraction of the O2−-forming NADPH oxidase is localized to the plasma membrane of the neutrophil (4, 8, 36). Activation of this oxidase can result in an extracellular release of oxygen metabolites, which could be of importance for the killing of nonphagocytized bacteria. In order to contribute effectively to the killing process without significant destruction of tissue components, the cellulyrly produced oxidative metabolites should be formed within the phagocytic vacuoles (24). When released extracellularly, the bactericidal molecules are highly toxic to the renal tissue (35).

In the present study we investigated the interaction between neutrophils and a number of E. coli strains isolated from patients suffering from pyelonephritis with and without renal scarring. The strains isolated from the scarred group gave rise to a more pronounced extracellular release of metabolites than did the strains isolated from the nonscarred group. The results suggest that the capacity of E. coli strains to cause extracellular neutrophil release of oxygen metabolites can be a key factor in the scarring process.

MATERIALS AND METHODS

Patients and bacterial strains. The 23 E. coli strains were selected from a larger group of previously described strains (30) that were isolated from girls with a history of recurrent acute pyelonephritis (more than two episodes). The subjects were all included in a urinary tract infection follow-up program with regular intervals of clinical and radiological investigations at the Division of Nephrology at the Children’s Hospital, Gothenburg, Sweden. The level of infection was determined by clinical, radiologic, and laboratory criteria (25). Acute pyelonephritis was diagnosed by the presence of significant bacteriuria in combination with a temperature >38°C, at least two abnormal laboratory findings of C-reactive protein (>20 mg/ml), and a microsedimentation rate ≥25 mm/h or a transiently decreased renal concentrating capacity. Vesicoureteric reflux (VUR) was diagnosed by means of voiding cystourethrogramy and graded 1 to 5 (23). Renal scarring was defined as calyceal deformity combined with parenchymal reduction on urography (21). The patients were divided into two groups, those with and those without renal scarring.

The bacterial strains isolated from the patients were first characterized according to the method of Edwards and Ewing (15) and then further analyzed for serotype, hemolysin production, sensitivity to the bactericidal effect of human serum, and binding properties (the relevant surface characteristics of the bacteria are summarized in Table 1). The strains were stored in deep agar slants, at 4°C, until used. For studies of interaction with neutrophils, the bacterial strains were grown on nutrient agar plates for 16 to 18 h and then transferred to liquid growth medium (tryptic soy broth) and grown overnight on a rotary shaker at 37°C. Thereafter
TABLE 1. Surface characteristics of the *E. coli* strains isolated from patients with acute pyelonephritis with or without renal scarring

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<sup>a</sup> MS, mannos sensitive; MR, mannos resistant.

the bacteria were harvested and washed three times in phosphate-buffered saline (pH 7.3) and resuspended (10⁹/ml) in Krebs-Ringer phosphate buffer supplemented with glucose (10 mM), Ca²⁺ (1 mM), and Mg²⁺ (1.5 mM) (KRG; pH 7.2).

Fluorescein isothiocyanate labelling of the bacteria was performed as described by Hed (20). The fluorescein isothiocyanate-labelling procedure does not affect cell viability or the physicochemical surface properties of the bacteria as measured with hydrophobic interaction chromatography and two-phase partitioning (unpublished observations).

Preparation of human polymorphonuclear neutrophils. To obtain normal neutrophils, heparinized blood from apparently healthy volunteers was separated by the method of Bøyum (6). After dextran sedimentation, hypotonic lysis, and Hypaque-Ficol centrifugation, the neutrophils were washed twice and resuspended to 2 × 10⁷ cells per ml in KRG and transferred to a melting ice bath. Samples were withdrawn from the cell suspension and used in the chemiluminescence (CL) or phagocytosis assay.

Phagocytosis system. The fluorescence technique described by Hed (20) was used to determine phagocyte-bacterium interaction. In short, the neutrophils (5 × 10⁴) were placed on multispot microscope slides and incubated for 20 min at 37°C in a humid environment. After the slides were washed, a bacterial suspension was added and the slides were incubated for another 30 min. The slides were washed and kept on ice until examined under the microscope. With this technique the total number of cell-associated bacteria (surface-adherent as well as internalized bacteria) is determined (20).

CL measurement. CL was measured in a six-channel Biolumat LB 9505 (Berthold, Wildbad, Germany) with disposable 4-ml polypropylene tubes. The tubes, each containing a 1.0-mI reaction mixture of KRG, neutrophils (2 × 10⁹), and luminol (5 × 10⁻⁶ M), were placed in the Biolumat and allowed to equilibrate for 5 to 10 min at 37°C. Two different reaction mixtures were used for quantification of intracellularly and extracellularly generated luminescence: the tubes were supplied with azide (1 mM) and hors eradish peroxidase (HRP; 4 U) for extracellularly generated CL, whereas superoxide dismutase (200 U) and catalase (2,000 U) were added to the tubes for the measurement of intracellularly generated CL. Bacterial suspension (100 μl) was added to activate the system, and the light emission was then recorded continuously.

Chemicals. Luminol was obtained from Sigma (St. Louis, Mo.). The scavengers catalase and superoxide dismutase as well as HRP (grade 1) were obtained from Boeringer Mannheim. NaOH (0.1 M) was used to dissolve luminol to 0.02 M. All substances were further diluted in KRG (pH 7.2).

Statistics. The Mann-Whitney-Wilcoxon sign rank test was used for statistical analysis.

RESULTS

Bacterial binding to the neutrophils. Binding of the *E. coli* strains to the neutrophils was measured by microscopic examination. The total number of cell-associated bacteria did not correlate with the known adhesin expression of the strains. Consistent with previous studies, most of the isolates expressing mannos-resistant adhesins showed a low degree of cell association. Even though the significance is low (P < 0.05), our present results indicate that the bacterial strains in the scarring group have an increased capacity for binding to neutrophils (Fig. 1).

CL response of neutrophils. Luminol-amplified CL was used to investigate the metabolic response induced in neutrophils upon interaction with the *E. coli* bacteria. Most of the bacterial strains induced such a response. However, the responses varied in both quantity and time course. The response induced by the scarring strains was not significantly higher than that elicited by the nonscarring strains, nor did the time to peak differ significantly between the two groups (data not shown).

Extracellular and intracellular CL activity. If the oxygen metabolites generated during neutrophil phagocytosis are to be restricted to the intracellular compartment, they should be produced after closing of the phagocytic vacuole. We have previously shown that luminol-amplified CL is dependent on the presence of the granule enzyme myeloperoxidase (12); this knowledge can be exploited to determine the relationship between intracellularly and extracellularly produced CL activity (7, 29). The detection system for the extracellular response contained azide (a low-molecular-weight myeloperoxidase inhibitor that gains access to intracellular compartments) combined with an azide-insensitive peroxidase (HRP). Azide alone totally inhibited the CL response, but in combination with HRP the response was regenerated. Since HRP is a large protein that does not have access to intracellular sites, it can be used to detect extracellularly released oxygen metabolites. The system for quantification of the intracellular response contained superoxide dismutase and catalase, large molecular scavengers of O₂⁻ and H₂O₂, respectively. In the presence of these scavengers the extracellularly produced O₂⁻ and H₂O₂ will be removed, thus providing a measurement of the intracellular response. In our experiments, the extracellular peak value was
always reached at an earlier time point than the intracellular peak. This suggested that adhesion of the bacteria to the phagocyte cell surface was associated with a greater leakage of oxygen metabolites than was the internalization. Representative curves of the response are shown in Fig. 2. There was no difference in the intracellular activity induced by the two groups of bacteria (Fig. 3a). Most strains belonging to the nonscarring group did not induce any extracellular activity, whereas most of the strains belonging to the scarring group did. We thus conclude that proportionally more extracellular activity was induced by the bacteria associated with scarring (Fig. 3b; P < 0.01).

**DISCUSSION**

We interpret the presented data to mean that there are qualitative differences in the ability to induce an extracellular release of reactive oxygen species from human neutrophils between uropathogenic *E. coli* bacteria isolated from patients with and without renal scarring.

Under circumstances ideal for the host, the interaction between neutrophils and invading bacteria should result in bacterial killing with minimal damage to host tissues. However, during neutrophil phagocytosis, the cellulary produced oxygen metabolites that are released inside the phagosome may leak out to the surrounding tissue, where they can inflict considerable damage (17). The techniques commonly used to measure the production of reactive oxygen metabolites cannot distinguish between intracellular and extracellular production (33). With the luminol-amplified CL technique, however, extracellular and intracellular events connected with a cellular response can be measured (3, 7, 11, 12, 19, 27, 29). The CL reaction is peroxidase dependent and totally inhibited by the myeloperoxidase inhibitor azide (12, 13, 16, 37). In addition, the *H₂O₂* scavenger catalase, the *O₂⁻* scavenger superoxide dismutase, and the azide-insensitive HRP are large proteins that have no access to intracellular sites; this means that the extracellular and intracellular CL responses can be detected separately (9-11, 27, 29). The majority of bacterial strains (both scarring and nonscarring) activated the neutrophils to generate oxygen radicals. Most of the activity induced by the nonscarring strains was limited to an intracellular compartment, possibly the phagosome, whereas the scarring strains induced a substantial extracellular release of the oxygen metabolites produced; this could be an effect of a lack of phagosome formation or vacuole closure. The ability of neutrophils to release oxygen metabolites could be important under conditions in which intracellular sequestering of bacteria is incomplete (28, 41), but the goal of killing must then be balanced against the threat made by the released metabolites on the host tissue.

The role of bacterial properties in the induction of renal scarring is a matter of debate (18, 21, 30, 40). The formation of scars has been assumed to result from the inflammatory tissue reactions during acute pyelonephritis (42). Bacterial properties which enhance the acute inflammatory response, e.g., binding to Galα1-4Galβ-containing receptors, would then be expected to increase the renal scarring frequency. However, strains from patients with renal scarring were rarely found to belong to the most uropathogenic *E. coli* clones (14, 30). The ability of *E. coli* to induce renal scars was thus suggested to be due to other unidentified virulence properties (40). This hypothesis was tentatively refuted by the finding that the scarring strains, as a group, lacked selectivity in terms of clonal identification markers. The findings of the present study provide the first suggestion for a mechanism whereby the scarring strains might have an increased propensity for attacking the renal tissue.

Neutrophil-*E. coli* interactions are initiated by binding of bacteria to the phagocyte membrane, a process that is
influenced by the surface characteristics of both the phagocyte and the prey (31). Binding is thus enhanced by bacterial lectins recognizing mannose residues (type I fimbriae) and fimbriae recognizing sialic acid residues (S fimbriae) (1, 32). Receptors for P fimbriae (i.e., the globoseries of glycolipids) are not abundant on human phagocytes (5, 38). Consistent with this, we show in the present study that most of the strains expressing mannose-resistant adhesins bound poorly to the phagocytes. There was no pattern in adhesin expression that could explain the bacterial binding to the neutrophils. However, although both the bacterial lipopolysaccharide and the capsular polysaccharide influence phagocyte-bacterium interactions, the differences between the scarring and nonscarring strains could not be explained by the serotype. Therefore, the bacterial determinant of extracellular release of oxidative metabolites remains to be identified.

The data concerning the relationship between inflammatory response and renal scarring are somewhat of a paradox. In experimental models, scarring can be caused by the acute inflammatory response (18, 40), a process that can be interrupted by treatment with antibiotics or anti-inflammatory agents (18, 26). In humans, however, bacteria which cause the highest acute inflammatory response (measured as fever and elevated C-reactive protein [CRP], erythrocyte sedimentation rate, and number of leukocytes in the urine) rarely cause renal scars (14, 30). This suggests that there may be qualitative differences between the inflammatory responses of patients with and without renal scarring. The results presented in this study indicate that the extracellular release of oxidative metabolites is a key to the scarring process. The fact that scavengers of oxidative metabolites can protect the renal tissue against the development of scars (34, 35) further strengthens this conclusion, but it should once again be pointed out not only that bacterial characteristics are of importance for the outcome of a bacterial phagocyte interaction but also that host defense could play a significant role. Thus, the role of phagocyte host factors for the oxygen metabolites released from phagocytes and for the damage to the human kidney inflicted by infection needs further investigation.

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