Cytotoxic Necrotizing Factor 1 Enhances Reactive Oxygen Species-Dependent Transcription and Secretion of Proinflammatory Cytokines in Human Uroepithelial Cells

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Uropathogenic *Escherichia coli* strains frequently produce a Rho-activating protein toxin named cytotoxic necrotizing factor type 1 (CNF1). We herein report that CNF1 promotes transcription and release of tumor necrosis factor alpha, gamma interferon, interleukin-6 (IL-6), and IL-8 proinflammatory cytokines and increases the production of reactive oxygen species (ROS) in uroepithelial T24 cells. The antioxidant *N*-acetyl-L-cysteine counteracts these phenomena, a fact which suggests a role for ROS-mediated signaling in CNF1-induced proinflammatory cytokine production.

Uropathogenic *Escherichia coli* strains, the most common bacterial cause of urinary tract infections, produce a number of virulence-associated factors, including aerobactin, hemolysin, P fimbriae, type 1 pili, and cytotoxic necrotizing factor type 1 (CNF1) (7, 8, 24, 29). CNF1 is a chromosomally encoded toxin that activates the small GTP-binding proteins of the Rho family (Rho, Rac, and Cdc42) by catalyzing their deamidation at a specific glutamine residue (19, 28, 35). As very recently reported by Doye and coworkers (12), CNF1 principally activates the Rac GTPase, and this activation is transient because the deamidated (activated) form of Rac is more susceptible to ubiquitin- and proteasome-mediated degradation. Degradation of the toxin-activated Rac switches on the cellular responses to CNF1 (12), which are largely dependent on the cell type (6). Recent *in vivo* studies reported that CNF1 plays an important role in establishing infection and promoting inflammation in the mouse bladder (32) and also contributes to *E. coli* virulence in a model of acute prostatitis (2, 33). This finding is in accordance with the generally accepted wisdom that infection invariably results in inflammation (31), which triggers, in turn, a rapid up-regulation of the transcription, synthesis, and release of proinflammatory cytokines (25). Although principally governed by specialized cells like macrophages, an inflammatory reaction can also be promoted by epithelial cells via mediators that transmit cellular signals to the immune system (22). The type of cytokine involved is rigorously defined by the strategy employed by the bacterium to interact with the host cell and by the nature of the two challengers (3, 23, 36). To investigate whether CNF1 contributes to the development of *E. coli*-driven inflammation, we tested the ability of the toxin to induce the transcription and secretion of proinflammatory cytokines in T24 cells, an epithelial cell line derived from human bladder tissue (American Type Culture Collection, Rockville, Md.) T24 cells were cul-

tured in McCoy's 5A medium supplemented with 10% fetal calf serum, nonessential amino acids, 100 µg of streptomycin per ml, and 100 U of penicillin per ml (all from GIBCO-BRL, Gaithersburg, Md.). CNF1 was obtained from the 392 ISS strain (kindly provided by V. Falbo, Rome, Italy) and purified as previously described (11, 13). Importantly, the CNF1 preparation was free of lipopolysaccharide (LPS), as determined by the Limulus assay (i.e., <0.03 U of LPS per ml). For all experiments, 2×10^4 cells/ml were seeded and 10^{-10} M CNF1 was added directly to the culture medium. Supernatant aliquots of T24 cells exposed to CNF1 for 3, 6, and 18 h were collected, and cytokines were measured by using commercially available enzyme-linked immunoabsorbent assay kits (R&D Systems). The cytokines investigated were those typically secreted by epithelial cells, and a closer look was taken at the cytokines stimulated by *E. coli* strains (1, 22). In particular, we analyzed gamma interferon (IFN-γ), a multifunctional cytokine first regarded as an antiviral factor; tumor necrosis factor alpha (TNF-α), a major mediator of inflammation; interleukin-6 (IL-6), a multifunctional cytokine with immunoregulatory and inflammatory effects; and IL-8, a potent chemoattractant for polymorphonuclear leukocytes.

As shown in Fig. 1a through d, although slight amounts of TNF-α, IFN-γ, IL-6, and IL-8 were appreciable even in the supernatant of untreated T24 cells, exposure to CNF1 significantly amplified their secretion. Specifically, the release of IFN-γ increased significantly in a time frame of toxin treatment ranging from 3 to 18 h (Fig. 1a), with a rapid decrease thereafter (data not shown). CNF1 also provoked a rapid increase in TNF-α secretion that peaked after 3 h of challenge with the toxin and then returned to the control cell value (Fig. 1b). IL-6 release increased with a constant trend along with the time of exposure to CNF1 (Fig. 1c), whereas the level of IL-8 secretion became significant after 6 h of treatment and reached a nearly fourfold higher value within 18 h (Fig. 1d). Moreover, to further rule out the possibility that the observed cytokine secretion could be due to LPS contamination (4, 34), a sample of CNF1 was heat inactivated by exposure of the protein toxin

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FIG. 1. CNF1 modulates cytokine release and transcription in T24 cells. (a to d) CNF1 induces a time-dependent release of cytokines as detected with commercial enzyme-linked immunoabsorbent assay kits. All samples were assayed in duplicate, and values are given as the means of the results from four separate experiments $\pm$ standard deviations. Student's *t* test for correlated samples was used, and a *P* value of less than 0.01 (*) was considered significant. (e to h) RT-PCR analysis reveals cytokine mRNA up-regulation after CNF1 exposure (IFN- γ [e], TNF- α [f], IL-6 [g], IL-8 [h]). Data represent values from one experiment representative of three performed. GAPDH cDNA levels were equivalent in all cell samples analyzed, as indicated. Results are expressed as arbitrary units (AU).

preparation to 98°C for 10 min; this procedure destroys protein but not LPS activity. No cytokine release was induced by the heat-inactivated CNF1 in T24 cells (data not shown). It is worth noting that the peak of TNF- α secretion (at 3 h) preceded those of IFN- γ (at 6 h) and IL-8 (at 18 h), thus confirming the role of TNF- α as a pluripotent activator of inflammation, capable of inducing a proinflammatory cytokine cascade (10). TNF- α , in fact, regulates many aspects of a host cell's defense mechanisms against pathogenic microbes, and its major effect is the activation of gene expression. Therefore, to analyze whether the augmented cytokine release correlated with an increase in mRNA transcription, T24 cells were treated with CNF1 for 3, 6, and 18 h and total cellular RNA was extracted by using the RNeasy kit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed as previously described (30) with the Access RT-PCR system (Promega) according to the manufacturer's instructions. A set of primers for IFN- γ , TNF- α , IL-6, and IL-8 was purchased from Clontech, while GAPDH (glyceraldehyde-3-phosphate dehydrogenase) sequences have been previously published (30). The reaction products were electrophoresed through a 1.8% agarose gel (Bio-Rad). Ethidium bromide-stained gels were scanned with a densitometer (Biomed Instruments), and cytokine mRNA levels were normalized to GAPDH mRNA levels (21). IFN- γ , TNF- α , IL-6, and IL-8 mRNA transcription was up-regulated within 3 h of CNF1 exposure (Fig. 1e through h). Notably, whereas IFN- γ , IL-6, and IL-8 mRNA transcription remained sustained until at least 18 h after CNF1 treatment, the levels of mRNA coding for TNF- α showed complete extinction after 18 h of treatment; these results are consistent with the cytokine secretion data reported above.

Increasing evidence suggests that reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide, can act as signaling intermediates for cytokine induction (15, 16). In order to investigate whether CNF1 could promote the production of cytosolic ROS, T24 cells exposed to the toxin for 30 min and for 1, 2, 3, and 6 h were stained for 15 min at 37°C with 5 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Sigma, St. Louis, Mo.), a fluorophore that measures intracellular ROS production (16). Both control and treated cells were immediately analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) equipped with a 15-mW, 488-nm-wavelength, air-cooled argon ion laser. In order to analyze only living cells, 5 μ M propidium iodide was added to all samples a few minutes before the acquisition of data. As shown in Fig. 2a, CNF1 increased cytosolic ROS in uroepithelial cells, which occurred in a time frame ranging from 1 to 3 h. It is worth noting that heat-inactivated CNF1 failed to augment the cytosolic ROS level in T24 cells (data not shown). To clarify whether this redox imbalance was responsible for the CNF1-mediated effects on cytokine production, additional experiments were carried out by using as an antioxidant the powerful thiol supplier *N*-acetyl-L-cysteine (NAC; Zambon Group, Milan, Italy) (9). NAC (10 mM) was directly dissolved in the culture medium and added to T24 cells for 2 h before the cells were exposed to CNF1. At this dose, the scavenger could counteract the toxin-raised ROS production (Fig. 2a), as described previously (17). NAC was also able to diminish CNF1-induced up-regulation of TNF- α , IL-6, and IL-8 gene expression (Fig. 2b), whereas IFN- γ seemed to be constitutively regulated by the redox state of the cell. Since Rac is one of the main promoters of cytosolic ROS production (5, 15), we can

FIG. 2. NAC counteracts ROS and cytokine production in CNF1-activated T24 cells. (a) Flow cytometry analysis reveals an increase in intracellular ROS in cells exposed to CNF1 for 3 h that was prevented by NAC. The histogram is representative of four separate experiments, and the values are expressed as median fluorescence intensities (MFI). (b and c) Bar charts indicate the inhibitory effect of NAC on CNF1-induced mRNA transcription (b) and cytokine release (c). Data in panels b and c represent the percentages of the ratio of CNF1-treated cells preincubated with NAC to CNF1-treated cells. Data in panel b exemplify values from one representative experiment of three performed and are expressed as arbitrary units; data in panel c result from assays performed in duplicate, and values, given in picograms per milliliter, are the means of the results of four separate experiments $\pm$ standard deviations.

hypothesize that the observed transcription of the cytokines follows the trend of Rac and ROS activation, increasing within 3 h of challenge with CNF1 and decreasing thereafter, when Rac is degraded (12). NAC is effective in impairing the CNF1-induced transcription of TNF- α , IL-6, and IL-8 at 3 h and, for IL-6 and IL-8, also at 6 h. This result can speculatively be explained by the ability of TNF- α to activate Rac once it is secreted, with Rac, in turn, possibly reactivating transcription

independently of ROS (27). Consequently, NAC failed to diminish TNF- α transcription at 6 h. The secretion of TNF- α conceivably mirrors the trend of transcription, being appreciable at 6 h but not at 3 h. The decrease induced by NAC at 6 h is most probably due to the hindrance of transcription that occurs earlier (at 3 h) rather than to blocked secretion. These data suggest that the molecular mechanisms utilized by ROS to transduce their message might involve oxidation of signal transduction molecules such as protein kinases and transcription factors. In order to verify whether the onset of intracellular ROS activity could also influence cytokine secretion, supernatant aliquots of T24 cells preincubated with NAC and then challenged with CNF1 for 3 and 6 h were analyzed. As illustrated in Fig. 2c, NAC had little effect on IL-6 production and the drop in IFN- γ secretion was independent of the toxin activity. In contrast, the antioxidant diminished TNF- α secretion (to 64%) and nearly abolished that of IL-8 (to 4%) after 6 h of CNF1 exposure. Of interest, IL-8 is constitutively produced in T24 cells (26), but its secretion occurs only after infection or transformation (20). From this result, we can hypothesize that the secretion of IL-8 strictly follows the trend of activation and degradation of Rac (12), whose proteosomal degradation switches on the cellular responses (12) and, most probably in this case, secretion. Thus, secretion of IL-8 starts to be evident at 6 h although its transcription (and most probably its presence inside the cell) is already detectable after 3 h (Fig. 1h).

All in all, these findings point toward the ability of CNF1-activated T24 cells to increase in a time-dependent manner the transcription and release of proinflammatory cytokines, such as TNF- α , IFN- γ , IL-6, and IL-8. This process involves cellular redox imbalance, with the transcription counteracted by the antioxidant NAC. Interestingly, NAC can also significantly impair the CNF1-induced release of IL-8, a master of the acute inflammatory response in the uroepithelium (32). Whereas high levels of ROS are normally produced by phagocytic cells as an essential mechanism of defense against invading microorganisms and are also responsible for injuries to host tissues, lower amounts are used by other cell types, such as epithelial cells, for signal transduction (16). Very recently, we have discovered that CNF1 may bring about a Rac-dependent superoxide anion release by epithelial cells, suggesting a novel role for epithelial cells in sharing with professional phagocytes the task of eliminating unwanted pathogens (14). In the present work, however, we have dealt with cytosolic ROS that may, at the low amounts detected, act as intracellular second messengers (15). Thus, CNF1 can be viewed as a crucial player in the urovirulence triggered by uropathogenic *Escherichia coli* strains, in accordance with in vivo studies reporting the contribution of the toxin to the inflammation and infection processes of uroepithelial tissues (2, 33). The ability of CNF1 to promote phagocyte-like behavior (13, 18) as well as an inflammatory reaction in epithelial cells may favor the spreading of bacteria through the host organism.

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