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Shiga toxin-1 regulation of cytokine production by human proximal tubule cells

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Background. Interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) levels are elevated in kidneys of patients with post-diarrheal hemolytic uremic syndrome (D+HUS) and may contribute to renal dysfunction. The renal cellular sources of these inflammatory cytokines in D+HUS are largely unknown, however, the proximal tubule has emerged as a potentially important candidate. Since Shiga toxin-1 (Stx-1) has been implicated in the genesis of D+HUS, we examined the effect of Stx-1 on cytokine production by human proximal tubule cells.

Methods. Stx-1 cytotoxicity, protein synthesis inhibition, and effect on IL-1, IL-6, and TNF protein release and mRNA levels were determined. The effect of another protein synthesis inhibitor, cycloheximide (CHX), on these parameters was also evaluated.

Results. Stx-1 greatly increased TNF release and mRNA levels while CHX, at concentrations that produced similar inhibition of protein synthesis, had no effect on TNF production. In contrast, Stx-1 and CHX caused comparable elevations in IL-1 release and mRNA accumulation. Stx-1 and CHX also stimulated IL-6 mRNA accumulation, but only at concentrations that either were cytotoxic or substantially blocked protein synthesis. Finally, lipopolysaccharide, which is likely to be elevated in the circulation of patients with D+HUS, had no effect alone, but synergized with Stx-1 to increase IL-1 production.

Conclusions. These results indicate that Stx-1 stimulates proximal tubule inflammatory cytokine production and that this effect is due partially to nonspecific induction of mRNA levels as well as activation of Stx-1-specific mechanisms.

Post-diarrheal hemolytic-uremic syndrome (D+HUS) is the leading cause of acute renal failure in children, and the disorder is characterized by acute renal injury, microangiopathic hemolytic anemia, and thrombocytopenia [1, 2]. D+HUS is associated with enteric infection by Shiga toxin (Stx) producing organisms (typically *E. coli* 0157:H7) [1, 2]. Stx binds to galactose-α-1,4, galactose-β-1,4, glucose-ceramide (GB_3), is internalized, and ultimately is cytotoxic by virtue of directly inhibiting peptide elongation [1]. The kidney expresses relatively high levels of GB_3 as compared to other organs, which may account, at least in part, for renal targeting in D+HUS [3]. This apparently straightforward scenario of renal damage in D+HUS is complicated, however, by observations suggesting that Stx may act in concert with other factors to elicit cellular dysfunction. Among these factors, inflammatory cytokines have emerged as being potentially important. These cytokines, including interleukins-1 (IL-1) and -6 (IL-6) and tumor necrosis factor (TNF), are secreted by macrophages in response to Stx [4]. Each of these proteins can elicit a plethora of well characterized inflammatory responses leading to cellular damage, renal thrombosis, fibrin accumulation, and vasoconstriction [1, 2]. In addition, IL-1 and TNF have been demonstrated to have a direct impact on Stx-mediated cytotoxicity: both cytokines induce expression of GB_3 on the surface of human umbilical and saphenous vein endothelial cells and up-regulate cell sensitivity to Stx cytotoxicity [5–7]. Taken together, these observations suggest that inflammatory cytokines play an important role in the pathogenesis of cellular dysfunction in the setting of D+HUS.

The cellular sources of inflammatory cytokines in D+HUS are largely unknown. Serum inflammatory cytokine levels in D+HUS have not been extensively studied and may not be a valid indicator of tissue cytokine production. Nonetheless, preliminary reports suggest IL-6 is elevated in the systemic circulation of these patients, while conflicting reports exist on whether serum TNF levels are increased [8–10]. Macrophages produce IL-1, IL-6, and TNF in response to Stx [4], however, macrophage infiltration in the kidney is not a prominent feature of D+HUS [2]. Interestingly, urinary excretion of TNF and IL-6 was very high during the acute phase of D+HUS [11], while inoculation of gnotobiotic mice with *E. coli* 0157:H7 increased renal TNF, IL-1, and IL-6 levels [12], suggesting that the kidney may produce inflammatory cytokines in

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D+HUS. This notion is supported by the observation that systemic administration of Stx to transgenic mice bearing a chloramphenicol acetyltransferase (CAT) reporter driven by the TNF promoter induced CAT activity primarily in the kidney [13]. These findings then beg the question that, if the kidney is a major source of cytokines in D+HUS, which renal cell(s) is/are involved? Studies directed at answering this question could be quite problematic; there are multiple cell types within the kidney that are potential sources of cytokine production. To narrow down the possibilities, it seemed reasonable, at least as a first assumption, to suspect those kidney cells that express relatively high levels of GB_3, exhibit relatively high Stx binding, and are highly responsive to the toxin. Studies to date indicate that proximal tubule cells may best meet these criteria [14]. First, Stx binding to normal human kidney sections is most prominent in renal cortical tubules [3, 15]. Second, urinary markers of proximal tubule damage are elevated early in the course of D+HUS [2]. Third, renal biopsies obtained early in the course of D+HUS reveal proximal tubule, as well as glomerular, damage [2, 16]. Fourth, since mice only develop tubulointerstitial disease following Stx injection, the finding that CAT reporter activity is substantially increased in mouse kidneys (although the proximal tubule was not specifically studied) after Stx administration suggests that renal tubules may be involved [13]. Finally, cultured human proximal tubule cells express very high levels of GB_3 and are exquisitely responsive to the toxin [14]. Taken together, these observations provide the basis for the first hypothesis to be tested in this study, namely, that Stx-induced increases in inflammatory cytokine production are due, at least in part, to toxin interaction with the proximal tubule.

An important consideration when studying Stx actions is that other bacterial-derived factors may contribute to cell injury in D+HUS. One such factor, lipopolysaccharide (LPS), is likely to be present in the blood of D+HUS patients [1, 2] and can markedly impair renal function. LPS alone does not cause HUS since the renal pathology in patients with gram negative septicemia does not resemble that of HUS [17]. LPS can, however, increase sensitivity to Stx by up-regulating cell surface GB_3 levels [6, 18]. Furthermore, LPS has been shown to stimulate cytokine release, including IL-6 and TNF, by mesangial and non-renal endothelial cells [19–22]. Hence, the second hypothesis tested in this study was that LPS acts in concert with Stx to modulate proximal tubule cell inflammatory cytokine production.

METHODS

Reagents

Human proximal tubule cells were obtained from Clonetics, Inc. (San Diego, CA, USA). Human IL-1β, IL-6, biotinylated goat anti-human IL-1β antibody (detection), and mouse anti-human IL-1β antibody (capture) from R&D Systems (Minneapolis, MN, USA); mouse anti-human TNF-α antibody (capture), biotinylated mouse anti-human TNF-α antibody (detection), rat anti-human IL-6 antibody (capture), and biotinylated rat anti-human IL-6 antibody from Pharmingen (San Diego, CA, USA); fetal calf serum was from Hyclone (Logan, UT, USA); random hexamers and *Taq* polymerase from Boehringer Mannheim (Indianapolis, IN, USA), RNAsin from Promega (Madison, WI, USA), deoxynucleotide triphosphates from Perkin-Elmer (Norwalk, CT, USA); ^{32}P-dCTP from Amersham (Arlington Heights, IL, USA); and horseradish peroxidase-Avidin D from Vector Laboratories (Burlingame, CA, USA). Penicillin/streptomycin, L-glutamine, insulin, transferrin, selenium, epidermal growth factor, murine Moloney Leukemia Virus reverse transcriptase, DMEM:F12, M199, and DMEM were purchased from Gibco (Grand Island, NY, USA). Unless stated otherwise, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Human proximal tubule cells were studied at the third passage. Proximal tubule identity and purity has previously been ascertained (Clonetics). Proliferating proximal tubule cultures were maintained in 1:1 Dulbecco's modified Eagle media:Ham's F12 (DMEM:F12) containing 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 5.5 mg/ml transferrin, 6.7 μg/ml selenium, 6.5 ng/ml L-thyroxine, 10 ng/ml epidermal growth factor, and 10% fetal calf serum at 37°C in a 5% CO_2 incubator. Barely confluent proximal tubule cell cultures were placed in DMEM:F12 containing only L-glutamine and penicillin/streptomycin for 24 hours prior to any experimental maneuvers. Only growth-arrested cells were examined since it was desirable to avoid any confounding influence of serum on cytokine release and since proliferating proximal tubule cells grow so fast that the cultures are greatly overconfluent within 48 hours of becoming barely confluent.

Cytotoxicity assay

Proximal tubule cultures in 96 well plates were exposed to 0.01 to 1 pg/ml Stx-1, 1 μg/ml LPS, 0.01 pg/ml Stx and 1 μg/ml LPS together, or 0.1 to 100 μM cycloheximide for 4, 24, or 48 hours. At the end of the incubation period, cells in 96 well plates were analyzed for neutral red uptake [23]. Cells were incubated in 50 μg/ml neutral red in M199 + 5% fetal bovine serum (serum necessary to reduce neutral red precipitation) for three hours at 37°C in a 5% CO_2 environment. Cells were rinsed in 1% formaldehyde and 1% $CaCl_2$ for 15 seconds, followed by addition of 50% ethanol and 1% acetic acid for 30 to 60 minutes. Absorbance was determined at OD_{450} (Molecular Devices ThermoMax

Microplate reader, Menlo Park, CA, USA), and the results were compared to control cells not exposed to toxin.

^{3}H-leucine uptake

Proximal tubule cultures in 24 well plates were exposed to 0.01 to 1 pg/ml Stx-1 or 0.1 to 100 μM cycloheximide for 4, 24, or 48 hours. Media was removed and HBSS containing 1 μCi/ml ^{3}H-leucine added for 20 minutes at room temperature. Cells were then rinsed with ice-cold HBSS and solubilized in 500 μl 0.1% sodium dodecyl sulfate. A 200 μl aliquot was incubated with 25 μl 200 mg/ml bovine serum albumin and 2 ml 10% tricarboxylic acid (TCA) for 60 minutes on ice, collected on pre-wetted GF/C filters (Whatman, Kent, UK) through a vacuum manifold, the filters washed with ice-cold 10% TCA and ice-cold 95% ethanol, and air dried. Filters were added to scintillation vials with 5 ml Ready Safe scintillation cocktail (Beckmann, Fullerton, CA, USA) and cpm determined with a Packard 2200CA beta-counter (Downers Grove, IL, USA).

Enzyme immunometric assay

Proximal tubule cultures in six well plates containing 2 ml of media were exposed to varying concentrations of Stx-1, 1 μg/ml LPS, LPS + Stx-1, or cycloheximide for 4, 24, or 48 hours. The supernatant was removed at the conclusion of the incubation times and aliquots used for determination of IL-1, TNF, and IL-6 levels by enzyme immunometric assay (EIA). EIAs were performed in 96 well plates in accordance with the Pharmingen General EIA Protocol. Empty wells were coated with 50 μl of 2 μg/ml of anti-IL-1β, anti-TNF-α, or anti-IL-6 capture antibodies in 50 mM Tris-HCl (pH 9.5) at 4°C overnight. Unbound antibody was rinsed away with 0.05% Tween 20 in PBS (pH 7.4) and nonspecific binding sites blocked by incubation with 200 μl 20% bovine calf serum in phosphate buffered saline (PBS) at 4°C overnight. After subsequent rinsing with PBS/Tween, standards or samples were added and incubated at 4°C overnight. Sample volume was generally 100 μl with the exception of 10 μl aliquots for IL-6 determination. Plates were then rinsed with PBS/Tween and 100 μl detection antibody added and incubated at room temperature (1 μg/ml biotinylated anti-TNF-α for 2 hr, 1 μg/ml biotinylated anti-IL-6 for 45 min, and 0.1 μg/ml biotinylated anti-IL-1β for 2 hr). The concentrations and incubation times of detection antibodies were empirically determined to yield optimal sensitivity and accuracy. Detection antibody was rinsed off using PBS/Tween and 100 μl 2.5 μg/ml horseradish peroxidase-Avidin D in 10% bovine calf serum in PBS added for 30 minutes at room temperature. Wells were rinsed with PBS/Tween and 100 μl 0.03% 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate buffer (pH 4.2) containing 0.03% H_2O_2 added for 10 minutes at room temperature. The reaction was stopped with 100 μl NaF and OD_{405} determined.

All antibodies used for EIA had no significant cross-reactivity with other cytokines for which they were not designed (including interleukins-1 to -8, TNF, transforming growth factor-β, platelet-derived growth factor, acidic and basic fibroblast growth factor, and colony stimulating factors). Anti-IL-1 and anti-TNF-α antibodies gave a linear detection between 5 and 5000 pg/ml, and anti-IL-1β antibodies gave a linear detection between 4 and 250 pg/ml.

The cells from which the supernatant was obtained were solubilized in 0.1 N NaOH. An aliquot of NaOH was mixed with Bradford reagent (Bio-rad, Richmond, CA, USA) and protein concentration determined by measuring absorbance at 590 nm [24]. All results are expressed as ng cytokine/mg total cell protein.

RNA analysis

Proximal tubule cultures in six well plates were exposed to varying concentrations of Stx-1, 1 μg/ml LPS, LPS + Stx-1, or cycloheximide for 4, 24, or 48 hours. Cells were then overlaid with 4 M guanidinium thiocyanate, 25 mM sodium citrate, 1% β-mercaptoethanol, and 1% sarcosyl (pH 7.0), aspirated several times through a 25 G needle, and RNA phenol/chloroform extracted and quantified spectrophotometrically [25]. A semiquantitative PCR method was employed for determination of ET-1 mRNA levels as previously described [26, 27]. Five micrograms of total RNA from each sample was reverse transcribed by incubating with 250 pmol random hexamers, 4 mM $MgCl_2$, 400 U murine Moloney Leukemia Virus reverse transcriptase, 80 U RNAsin, 500 μM deoxynucleotide triphosphates (dNTP), 1 mM dithiothreitol, 50 mM KCl, 10 mM Tris-Cl, and 0.01% gelatin (final buffer pH 8.3) in 50 μl for one hour at 37°C. The reverse transcriptase was inactivated by heating for 10 minutes at 94°C. The resultant cDNA was amplified by polymerase chain reaction. Each sample was measured for IL-1β, TNF-α, IL-6, and β-actin cDNA in separate tubes using specific primers (Fig. 1). The upstream and downstream primers for IL-1β were CCATCACAGGTAGTGAGACCAACC and CCATAGTCAGTAGCTCTGGTCCTC, respectively. These yielded a single 449 base pair fragment which, when cycle sequenced with fluoresceinated primer ends (performed by Margaret Robinson in Dr. Ray White's laboratory at the University of Utah), proved identical to positions 696 to 1145 in human IL-1β cDNA. The upstream and downstream primers for TNF-α were GGTGACCGACTCAGCGCTGAGATC and GGTTGCCAGCACTTCACTGTGCAG, respectively, which yielded a 299 base pair product identical to positions 698 to 997 in human TNF-α cDNA. The upstream and downstream primers for IL-6 were ACATCCTCGACGGCATCTCAG and TGGCTTGTTCCTCACTACTCT, respectively, which yielded a 246 base pair product complementary to positions 262 to 507 in human IL-6 cDNA. Omission of reverse transcriptase prior to PCR using primers for IL-1β, TNF-α, or IL-6 failed to yield any

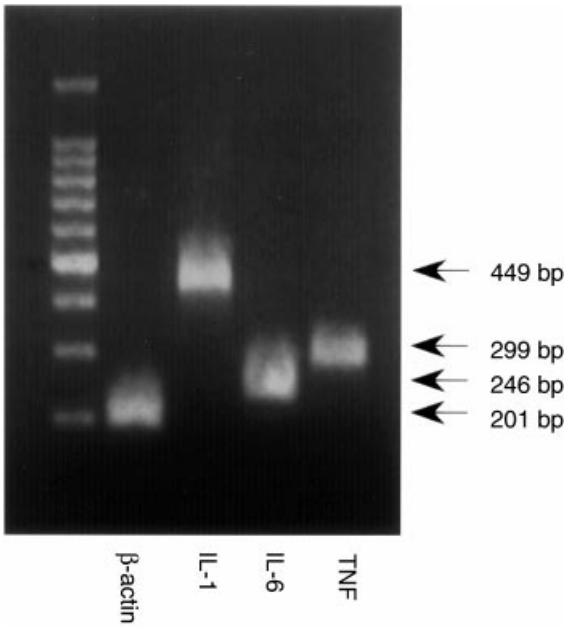

Fig. 1. Representative blot of polymerase chain reaction (PCR) products for β-actin, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF).

product. The upstream and downstream primers for β-actin were TGGAGAAGAGCTATGAGCTGCCTG and GTGCCACCAGACAGCACTGTGTTG, respectively, which yielded a 201 base pair cDNA fragment. PCR of genomic DNA yielded a 289 base pair product that is complementary to positions 2499 to 2788 in the β-actin gene, confirming that this primer set spans an intron.

Polymerase chain reaction (PCR) was performed by incubating 5 μl (approximately 0.4 μg) of sample cDNA with 50 mM KCl, 10 mM Tris-Cl, 0.01% gelatin, 1.5 mM $MgCl_2$, 2.5% formamide, 2 U *Taq* polymerase, 200 μM each dNTP, 100 pmoles of primers, and 0.1 μCi ^{32}P-dCTP in 50 μl final volume (final pH 8.3 at room temperature). PCR using β-actin, IL-6, IL-1β, or TNF-α primers were carried out for 20, 30, 35 or 35 cycles, respectively, using a Perkin-Elmer Cetus 9600 Gene-Amp System. This number of cycles result in samples being obtained during the exponential phase of amplification. Cytokine and β-actin primers were never combined in the same tube. Twenty microliters of the final PCR reaction was electrophoresed on a 7% non-denaturing polyacrylamide gel. Gels were stained with ethidium bromide and the bands corresponding to the cDNA product were excised, mixed with scintillation cocktail, and cpm determined on a Beckman beta counter. A representative gel is shown in Figure 1.

Cytokine and β-actin cDNA obtained from PCR of reverse transcribed RNA were used to generate standard curves. The cDNA was amplified by PCR, the resultant amplified product divided into small fractions that were, in turn, re-amplified. After removal of primers using Magic PCR Prep (Promega), the purity of the final product was confirmed by electrophoresis. At the end of the purification, the amount of standard cDNA was quantitated spectrophotometrically. Standard curves for β-actin or cytokines were made by simultaneously amplifying sample cDNA and, in separate tubes, standard cDNA (10^{-1} to 10^{-8} ng/tube). Every PCR amplification included a standard curve. All PCR consisted of simultaneous amplification (in separate tubes) of cDNA for cytokines and β-actin. All results are expressed as fg cytokine cDNA/ng β-actin cDNA in order to control for the amount of RNA initially reverse transcribed. The accuracy of this semiquantitative PCR technique has been previously described in detail [26–28].

Purification of Shiga toxin-1

Shiga toxin-1 was purified as previously described [14] from *E. coli* HB101 containing pNAS13 (generous gift of Alison D. O'Brian, Uniformed Services University of the Health Sciences, Bethesda, MD, USA), a derivative of pNAS4 [29] that contains a 3.4 kb NcoI fragment encoding Stx-1. The crude toxin preparation from bacterial lysates was dialyzed against 50 mM Tris-HCl (pH 8), subjected to CL-6B DEAE-Sepharose (Pharmacia, Uppsala, Sweden) anion-exchange chromatography using the same buffer, and eluted with a 0 to 0.5 M NaCl gradient in the same buffer. Fractions were pooled based on their cytotoxicity to Vero cells and dialyzed against phosphate-buffered saline (PBS), pH 7.4. Crude toxin was subjected to immunoaffinity chromatography with a monoclonal antibody to the B-subunit of Stx-1 (13C4; ATCC, Rockville, MD, USA) linked to an AminoLink column (Pierce, Rockford, IL, USA). Eluted samples were tested for cytotoxicity on Vero cells and protein directly visualized by electrophoresis on 15% native and denaturing polyacrylamide gels. Cytotoxic fractions were combined, concentrated and dialyzed against PBS (pH 7.4). LPS contamination was non-detectable using an E-Toxate assay (Sigma). Toxin concentration was based on spectrophotometric O.D. and Bradford protein assay.

Statistics

All data were analyzed by one way analysis of variance. Results are expressed as mean ± SEM. P values < 0.05 were taken as significant.

RESULTS

Effect of Shiga toxin-1 on proximal tubule cytotoxicity and protein synthesis

Cytotoxicity was assessed by a combination of neutral red, total cell protein, and cell morphology. Total cell protein paralleled neutral red data, however, it was not as sensitive in detecting cell survival and thus is not included in the cytotoxicity analysis. Alterations in cell morphology

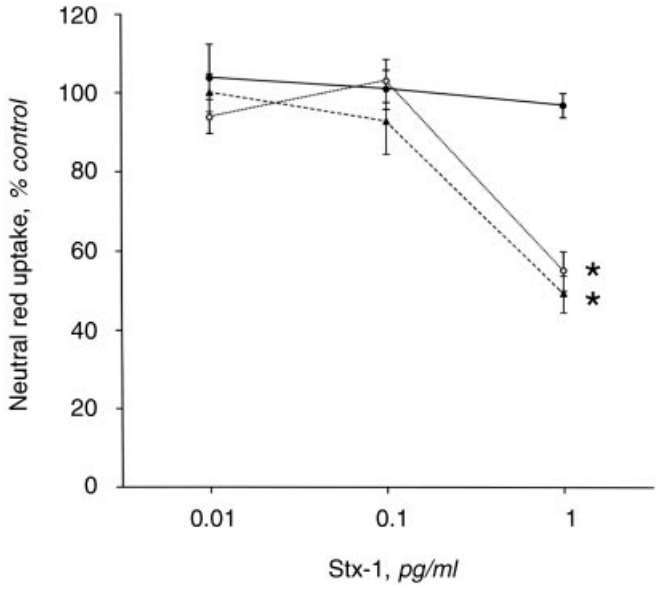

Fig. 2. Effects of Shiga toxin-1 (Stx-1) on human proximal tubule cell viability. The lower the neutral red uptake, the greater the cell death. $N = 12$ each data point. *P < 0.001 versus control. Symbols are: (●) 4 hr; (○) 24 hr; (▲) 48 hr.

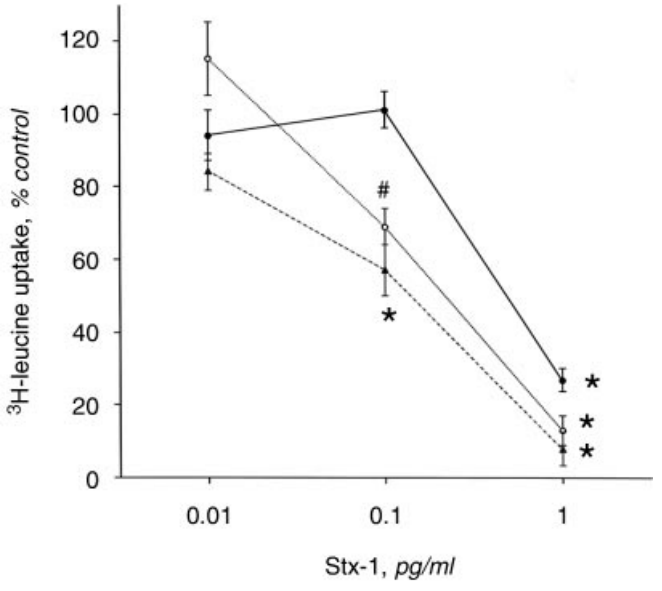

Fig. 3. Effect of Stx-1 on human proximal tubule cell ^{3}H-leucine uptake (measure of protein synthesis). $N = 3$ each data point. *$P < 0.001$ versus control; #$P < 0.005$ versus control. Symbols are: (●) 4 hr; (○) 24 hr; (▲) 48 hr.

generally agreed with neutral red uptake data: marginally toxic concentrations of Stx-1 mildly increased cytoplasmic granularity, while higher toxin concentrations gradually increased cytoplasmic granule number and size, caused irregular cell borders, cell flattening, and ultimately led to cell detachment.

Shiga toxin-1-induced alterations in proximal tubule cytokine release could occur by two major mechanisms: (*1*) Stx-1-specific modulation of signaling pathways; or (*2*) nonspecific effects resulting from cell cytotoxicity and/or protein synthesis inhibition. To help discriminate between these two possibilities, a dose- and time-response for Stx-1-induced proximal tubule toxicity was determined. As illustrated in Figure 2, up to 48 hours incubation with 0.01 to 0.1 pg/ml Stx-1 had no detectable effect on proximal tubule cell viability, hence these conditions were initially identified to study "non-cytotoxic" effects of Stx-1. In contrast, 24 to 48 hours exposure to 1 pg/ml Stx-1 caused a 50% reduction in cell survival, and these conditions were used to study cytokine release under "cytotoxic" concentrations of Stx-1. Higher concentrations of Stx-1 (≥10 pg/ml) caused over 90% cell death after 24 hours [11] and were, therefore, not used in the current study. Finally, the effect of LPS on cell survival was examined. LPS had no cytotoxic effect alone or in combination with 0.01 pg/ml Stx-1 (data not shown).

The Stx-1 concentrations and time courses selected above included those that were grossly non-cytotoxic; however, the possibility remained that substantial protein synthesis inhibition occurred under these "non-cytotoxic" conditions. This raised the possibility that Stx-1 effects, while not associated with reduced cell viability, could be a nonspecific response to reduced protein synthesis. To help examine this issue, the effect of Stx-1 on ^{3}H-leucine uptake, a measure of protein synthesis, by human proximal tubule cells was studied. As demonstrated in Figure 3, exposure to 0.01 pg/ml Stx-1 for up to 48 hours had no detectable effect on ^{3}H-leucine uptake. Higher concentrations of the toxin, particularly after 24 hours exposure, significantly reduced ^{3}H-leucine incorporation into protein. Taken together with the toxicity studies above, these findings indicated that a range of Stx-1 concentrations and exposure times of 0.01 to 1 pg/ml and 4 to 48 hours, respectively, would cover the spectrum from no detectable effect on cell survival or protein synthesis to frankly cytotoxic doses. By studying Stx-1 effects under this range of conditions, our ability to discriminate between specific and nonspecific effects of Stx-1 should be enhanced.

Effect of cycloheximide on proximal tubule cytotoxicity and protein synthesis

To further evaluate the specificity of Stx-1 action, the effect of cycloheximide (CHX), another protein synthesis inhibitor, on inflammatory cytokine release by proximal tubule cells was also determined. Similar to Stx-1, it was first necessary to identify the dose- and time-dependence of CHX cytotoxicity and protein synthesis inhibition. As shown in Figure 4, CHX concentrations from 0.1 to 100 μM over 4 to 48 hours achieved variable proximal tubule cell cytotoxicity (as determined by neutral red uptake), ranging from no detectable effect (4 hr or 0.1 μM CHX) to approximately 60% cell death. The same CHX exposure

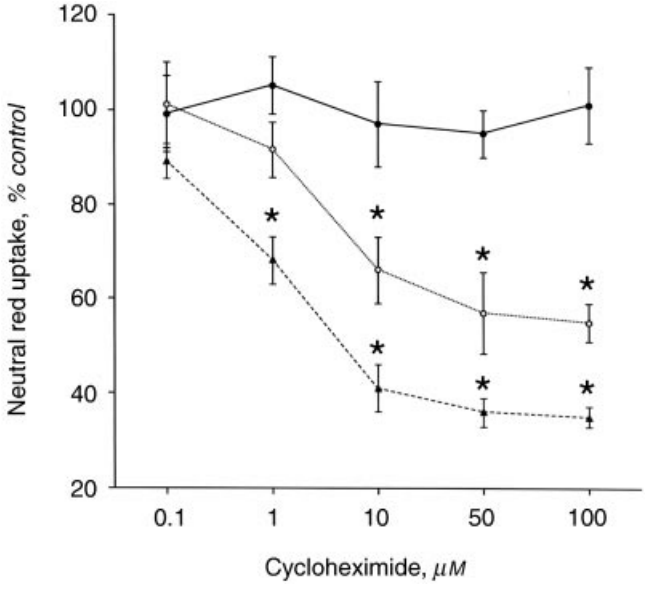

Fig. 4. Effect of cycloheximide on human proximal tubule cell viability. The lower the neutral red uptake, the greater the cell death. $N = 12$ each data point. $^{*}P < 0.001$ versus control. Symbols are: (●) 4 hr; (○) 24 hr; (▲) 48 hr.

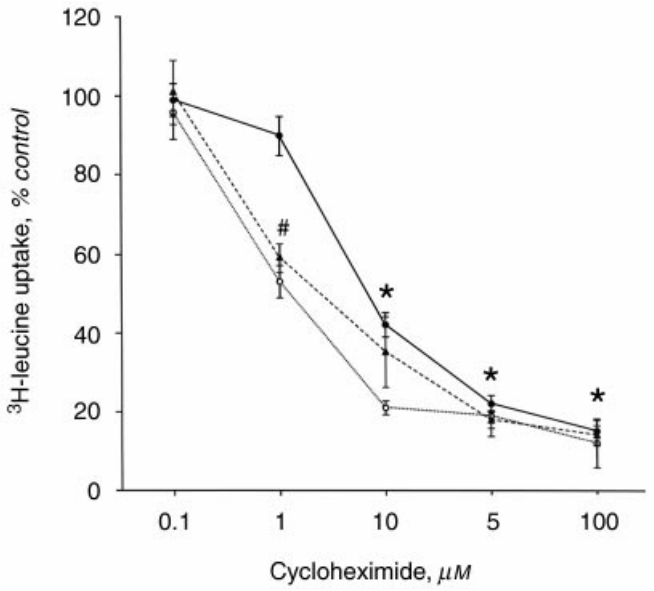

Fig. 5. Effect of cycloheximide on human proximal tubule cell ^{3}H-leucine uptake (measure of protein synthesis). $N = 3$ each data point. $^{*}P < 0.001$ versus control for all time points at the indicated dose; $^{\#}P < 0.001$ for 24 and 48 hours time points at the indicated dose. Symbols are: (●) 4 hr; (○) 24 hr; (▲) 48 hr.

times and doses also achieved variable inhibition of protein synthesis (as determined by ^{3}H-leucine uptake) ranging from no measurable effect (0.1 μM CHX) to near complete blockade (Figure 5). Thus, 4 to 48 hours exposure to 0.1 to 100 μM CHX was used in the current study to compare the effect of another protein synthesis inhibitor and cytotoxic agent to that of Stx-1.

Shiga toxin-1 and cycloheximide regulation of cytokine release and mRNA levels

Tumor necrosis factor. Proximal tubule cells released 84.2 ± 17.4 ng TNF/mg total cell protein/24 hr under basal conditions; Stx-1 augmented TNF release approximately two- to fourfold (note that all data points are compared to controls, not to one another; Fig. 6A). This effect occurred primarily at non-cytotoxic concentrations of Stx-1, however, augmented TNF release was generally associated with toxin concentrations that inhibited protein synthesis. It should be noted that exposure to 0.01 pg/ml Stx-1 for 24 to 48 hours increased TNF release without detectably altering protein synthesis or cell survival.

Proximal tubule cells contained baseline TNF mRNA levels of 0.12 ± 0.03 fg TNF cDNA/ng β-actin cDNA. Stx-1 stimulated TNF release was associated with augmented TNF mRNA levels (Fig. 6B). The increase in TNF mRNA was of a greater magnitude (when expressed as percent of control) than was the rise in TNF release; this dissociation was most evident at the highest toxin concentration. Presumably this reflects, at least in part, the effect of combined stimulation of mRNA accumulation coincident with partial inhibition of protein synthesis (see **Discussion**).

Since Stx-1 stimulation of TNF release and mRNA accumulation occurred mainly at toxin concentrations that partially inhibited protein synthesis, it was of interest to compare the effect of CHX on TNF production. As shown in Figure 7, CHX did not change TNF release or mRNA accumulation, even at concentrations that were frankly cytotoxic and substantially blocked protein synthesis. Hence, it is clear that Stx-1 stimulation of TNF secretion and mRNA levels is a specific phenomenon and not solely a result of global protein synthesis inhibition or cellular injury.

Interleukin-1.

Proximal tubule cells released 9.0 ± 1.4 ng IL-1/mg total cell protein/24 hr under basal conditions, and Stx-1 elevated IL-1 secretion two- to fourfold (again, note that all data points were compared to controls, and not to one another; Fig. 8A). As for TNF, Stx-1 stimulation of IL-1 release occurred primarily at toxin concentrations that were non-cytotoxic but did inhibit protein synthesis. Again similar to TNF, exposure to 0.01 pg/ml Stx-1 for 24 hours increased IL-1 release without detectably altering protein synthesis or cell survival.

Proximal tubule cells contained baseline IL-1 mRNA levels of 0.66 ± 0.34 fg IL-1 cDNA/ng β-actin cDNA. Stx-1 stimulated IL-1 secretion was associated with elevated IL-1 mRNA levels (Fig. 8B). Like TNF, there was a relatively greater rise in IL-1 mRNA (when expressed as percent of control) as compared to the rise in IL-1 release, particularly at toxin concentations that substantially inhibited protein

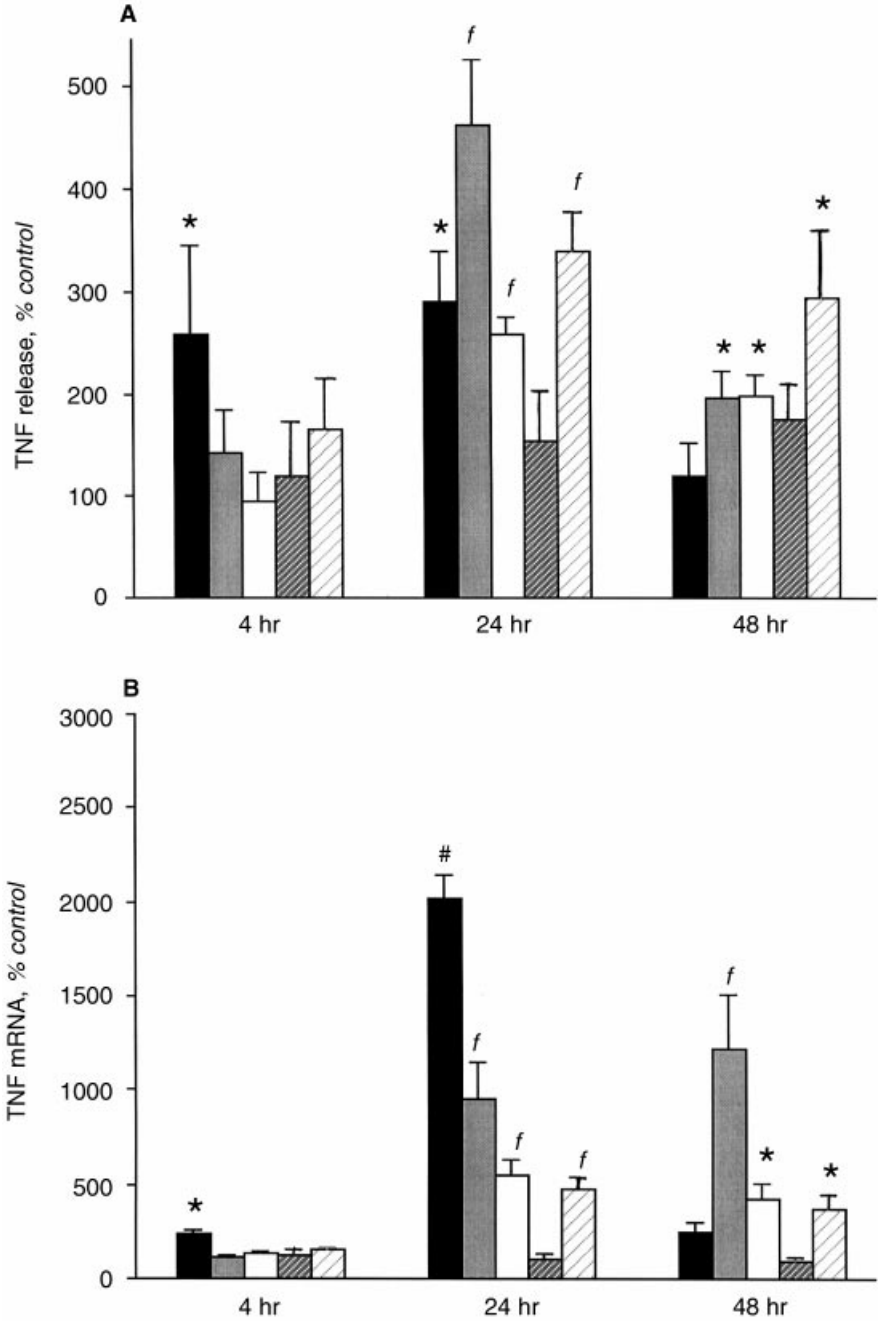

Fig. 6. Effect of Shiga toxin-1 (Stx-1) ± lipopolysaccharide (LPS) on human proximal tubule cell tumor necrosis factor (TNF) release (*A*) and TNF mRNA levels (*B*). Results are expressed as percent of control where baseline TNF release was 84.2 ± 17.4 ng TNF/mg total cell protein/24 hours and baseline TNF mRNA levels were 0.12 ± 0.03 fg TNF cDNA/ng β-actin cDNA. $N = 6$ per TNF release data point and $N = 3$ per TNF mRNA data point. $^{*}P < 0.05$, $^{f}P < 0.01$, $^{\#}P < 0.001$; all versus control. Symbols are: (■) Stx-1 1 pg/ml; (▨) Stx 0.1 pg/ml; (□) Stx-1 0.01 pg/ml; (▨) LPS; (▨) LPS/Stx-1 0.01 pg/ml.

synthesis. This dissociation is most evident with exposure to 1 pg/ml Stx-1 for 48 hours where IL-1 mRNA was induced 25-fold, but IL-1 release was not elevated. Under these conditions, Stx-1 was frankly cytotoxic as well.

Stx-1 stimulation of IL-1 release and mRNA accumulation occurred primarily at toxin concentrations that reduced protein synthesis. To evaluate the specificity of this response, the effect of CHX on IL-1 production by proximal tubule cells was determined. Like Stx-1, CHX increased IL-1 release and mRNA levels (Fig. 9). Also like Stx-1, CHX stimulation of IL-1 release mRNA accumulation occurred mainly at concentrations that reduced protein synthesis. Thus, unlike clearly different patterns of regulation of proximal tubule cell TNF production, the patterns of Stx-1 and CHX modulation of proximal tubule cell IL-1 production appeared to be similar.

Interleukin-6. Proximal tubule cells released large amounts of IL-6 (9566 ± 580 ng IL-6/mg total cell protein/24 hr under basal conditions) and the basal secretion rate was approximately two- to three orders of magnitude greater than that seen with IL-1 or TNF. Proximal tubule cells also expressed relatively high levels of IL-6 mRNA

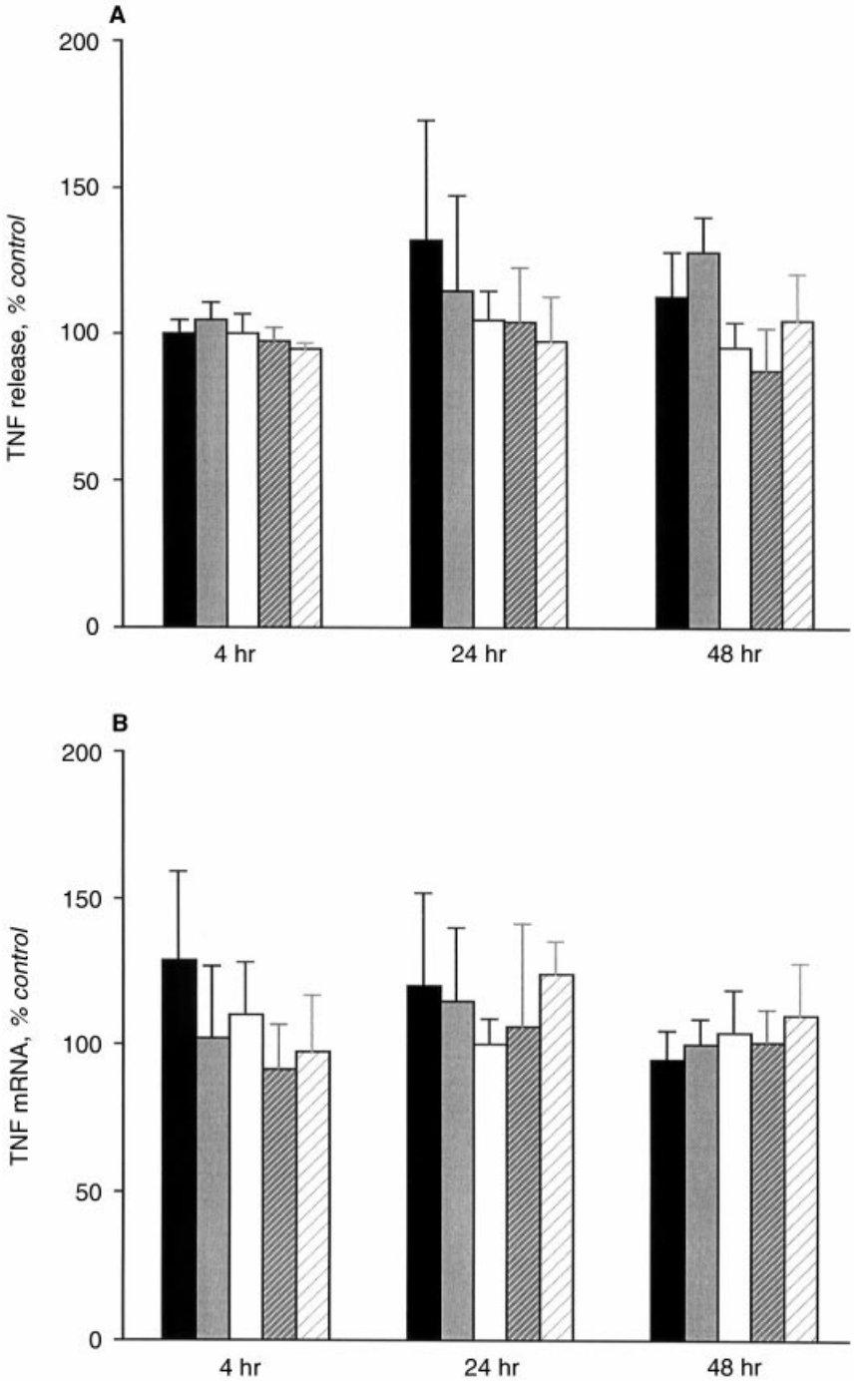

Fig. 7. Effect of cycloheximide on human proximal tubule cell TNF release (*A*) and TNF mRNA levels (*B*). Results are expressed as percent of control where baseline TNF release was 84.2 ± 17.4 ng TNF/mg total cell protein/24 hr and baseline TNF mRNA levels were 0.12 ± 0.03 fg TNF cDNA/ng β-actin cDNA. $N = 6$ per TNF release data point and $N = 3$ per TNF mRNA data point. Symbols are: (■) 100 μM; (▩) 50 μM; (□) 10 μM; (▨) 1 μM; (▨) 0.1 μM.

under baseline conditions: 6.64 ± 2.75 fg IL-6 cDNA/ng β-actin cDNA. Stx-1 had no effect on IL-1 release (as above, all data points were compared to controls, and not to one another; Fig. 10A), although the toxin did enhance IL-6 mRNA accumulation (Fig. 10B). The increase in IL-6 message only occurred at toxin concentrations that inhibited protein synthesis. Furthermore, IL-6 mRNA accumulation was greatest when frank cytotoxicity was evident (1 pg/ml Stx-1 at 24 and 48 hr time points). It is also important to note that Stx-1 stimulation of IL-6 mRNA accumulation required toxin concentrations and exposure times that were greater than those necessary to augment IL-1 or TNF production.

The effect of CHX on proximal tubule cell IL-6 release was also determined (Fig. 11). Like Stx-1, CHX had no effect on IL-6 release. CHX did enhance IL-6 mRNA accumulation, however, this only occurred under severe conditions associated with marked cell death and protein

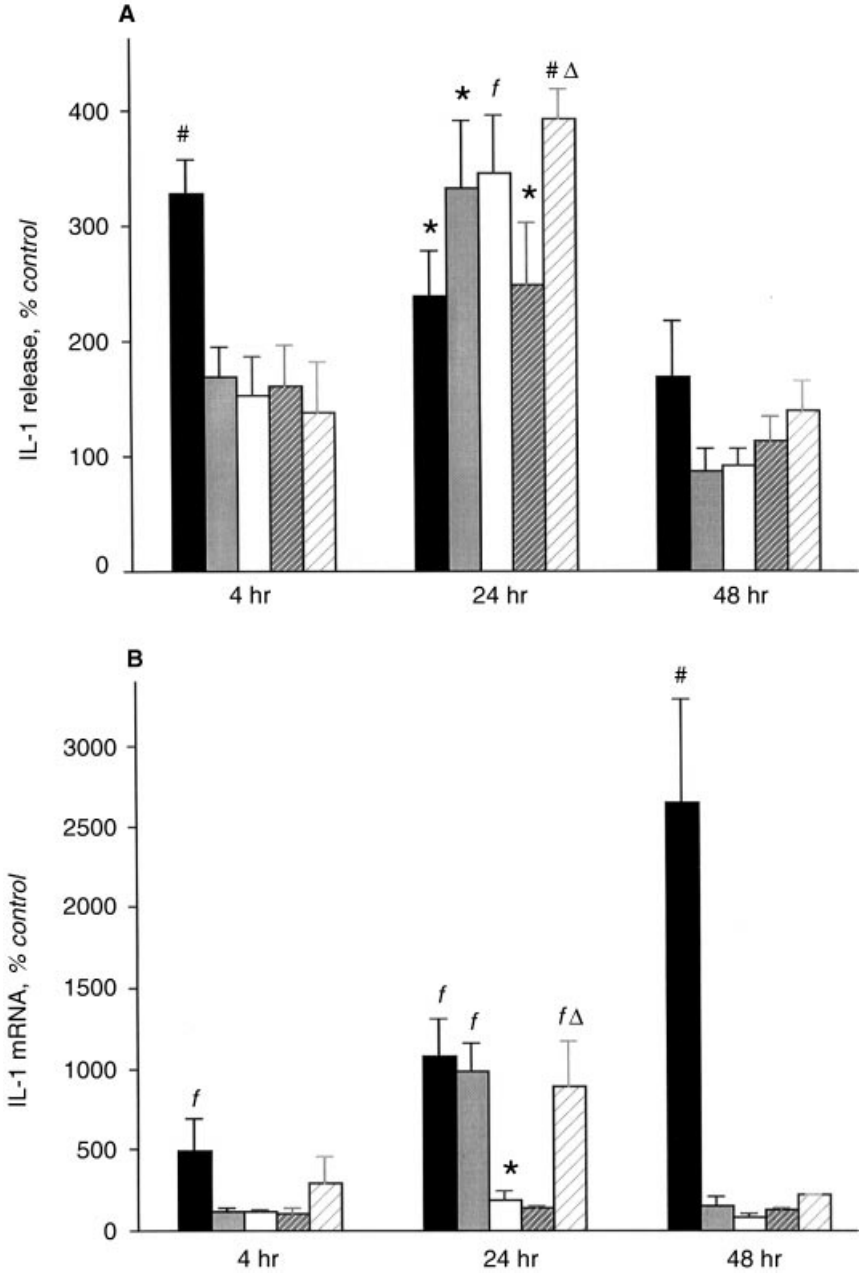

Fig. 8. Effect of Stx-1 ± LPS on human proximal tubule cell IL-1 release (*A*) and IL-1 mRNA levels (*B*). Results are expressed as percent of control where baseline IL-1 release was 9.0 ± 1.4 ng IL-1/mg total cell protein/24 hr and baseline IL-1 mRNA levels were 0.66 ± 0.34 fg IL-1 cDNA/ng β-actin cDNA. $N = 6$ per IL-1 release data point and $N = 3$ per IL-1 mRNA data point. $^{*}P < 0.05$, $^{f}P < 0.01$, $^{\#}P < 0.001$; all versus control. $^{\Delta}P < 0.05$ versus 0.01 pg/ml Stx-1 alone. Symbols are: (■) Stx-1 1 pg/ml; (▨) Stx 0.1 pg/ml; (□) Stx-1 0.01 pg/ml; (▨) LPS; (▨) LPS/Stx-1 0.01 pg/ml.

synthesis inhibition. Thus, CHX modulation of IL-6 production seems to be grossly similar to that with Stx-1 in that the cells appear to be dead or dying.

Effect of lipopolysaccharide on cytokine release and mRNA levels

Since LPS is likely to be elevated in the circulation of patients with HUS, and since the endotoxin has been demonstrated to stimulate inflammatory cytokine production by some cell types, the effect of LPS ± Stx-1 on proximal tubule cell IL-1, IL-6, and TNF production was evaluated. LPS had no effect on basal or Stx-1 (0.01 pg/ml) regulated IL-6 (Fig. 10) or TNF (Fig. 6) production. LPS also had no effect on basal IL-1 production, although the endotoxin did significantly enhance Stx-1-induced IL-1 release and mRNA accumulation (Fig. 8). This effect was, however, only evident after 24 hours exposure to LPS and Stx-1. Finally, it should be noted that the LPS concentration (1 μg/ml) was selected on the basis of its consistent stimulation of responses in a wide variety of cell types [17].

DISCUSSION

The current study examined the effects of Stx-1 and LPS on IL-1, IL-6, and TNF release by, and mRNA levels in, cultured human proximal tubule cells. Several potentially

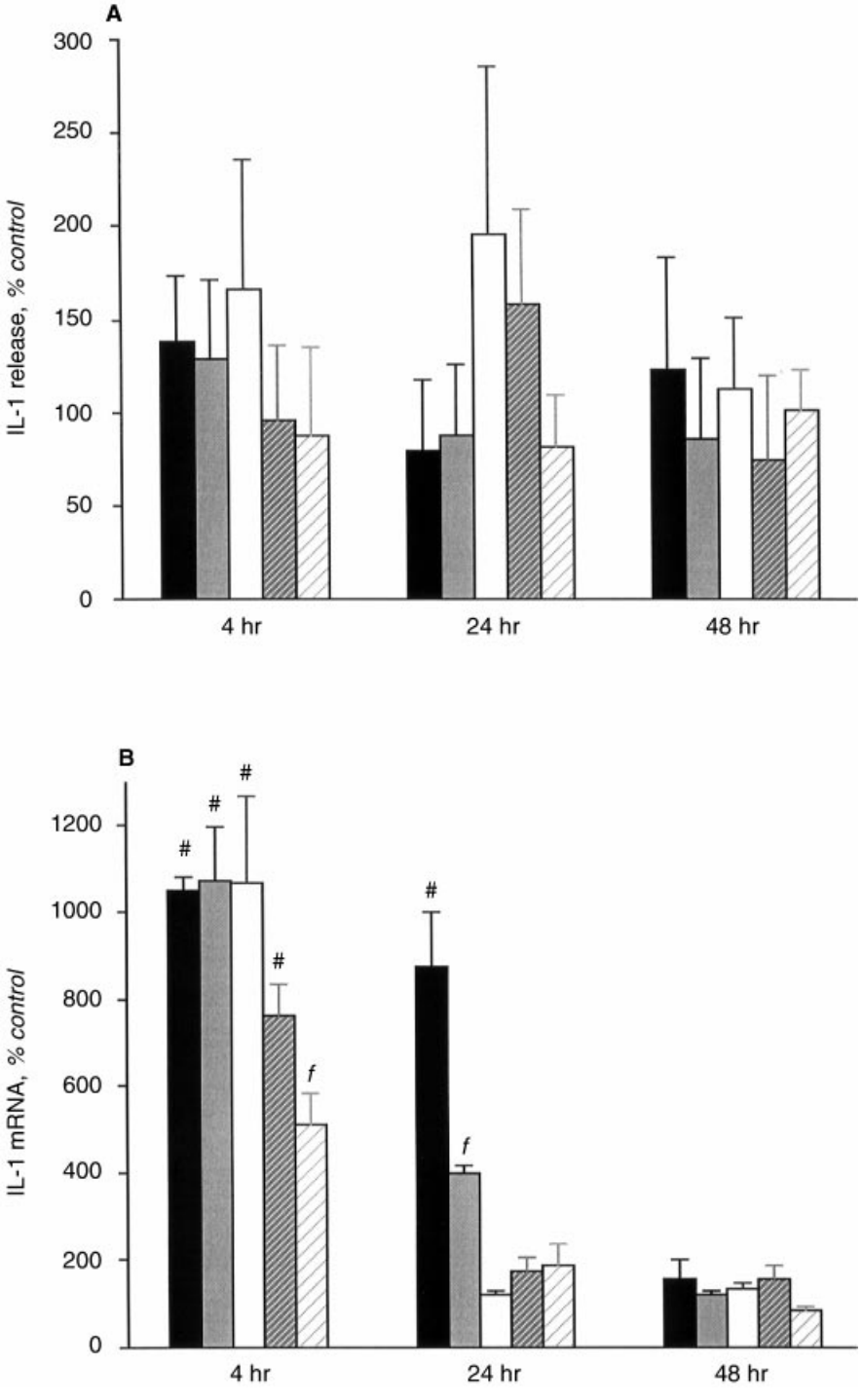

Fig. 9. Effect of cycloheximide on human proximal tubule cell IL-1 release (*A*) and IL-1 mRNA levels (*B*). Results are expressed as percent of control where baseline IL-1 release was 9.0 ± 1.4 ng IL-1/mg total cell protein/24 hr and baseline IL-1 mRNA levels were 0.66 ± 0.34 fg IL-1 cDNA/ng β-actin cDNA. $N = 6$ per IL-1 release data point and $N = 3$ per IL-1 mRNA data point. $^{*}P < 0.05$, $^{f}P < 0.01$, $^{\#}P < 0.001$; all versus control. Symbols are: (■) 100 μM; (▨) 50 μM; (□) 10 μM; (▨) 1 μM; (▨) 0.1 μM.

important observations resulted from this study: (*1*) human proximal tubule cells synthesize IL-1, IL-6, and TNF; (*2*) Stx-1 increases inflammatory cytokine production by these cells; (*3*) the mechanism of Stx-1 stimulated inflammatory cytokine production differs between IL-1, IL-6, and TNF; and (*4*) LPS enhances Stx-1 stimulated IL-1 production.

We confirm previous reports that human proximal tubule cells produce IL-6 and TNF [30, 31] and provide the new observation that IL-1 is also made by this cell type. The relative quantities of inflammatory cytokine release by proximal tubules has not previously been studied to our knowledge. In the current study, IL-6 was released in 1000-fold greater amounts than TNF and 100-fold greater amounts than IL-1. The increased IL-6 production was paralleled by 10- to 50-fold greater IL-6 than IL-1 or TNF mRNA levels. The reason for such a relatively high baseline

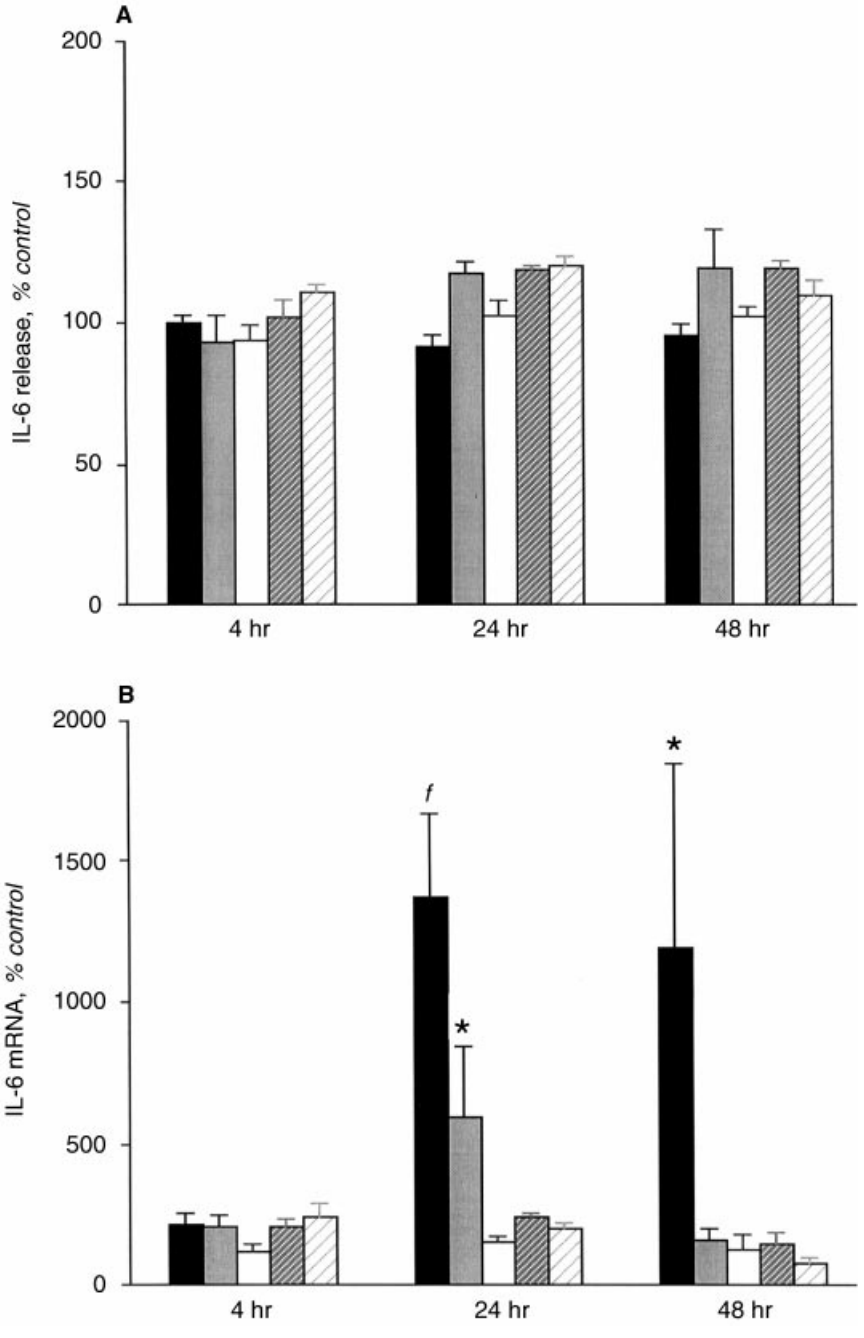

Fig. 10. Effect of Stx-1 ± LPS on human proximal tubule cell IL-6 release (*A*) and IL-6 mRNA levels (*B*). Results are expressed as percent of control where baseline IL-6 release was 9566 ± 580 ng IL-6/mg total cell protein/24 hr and baseline IL-6 mRNA levels were 6.64 ± 2.75 fg IL-6 cDNA/ng β-actin cDNA. $N = 6$ per IL-6 release data point and $N = 3$ per IL-6 mRNA data point. $^{*}P < 0.05$, $^{f}P < 0.01$; both versus control. Symbols are: (■) Stx-1 1 pg/ml; (▩) Stx 0.1 pg/ml; (□) Stx-1 0.01 pg/ml; (▨) LPS; (▨) LPS/Stx-1 0.01 pg/ml.

production of IL-6 is uncertain. It is possible that these cells are somehow modified during the culture process so that they produce greater amounts of IL-6 than seen *in vivo*. It is unlikely, however, that proximal tubule cells are globally activated during culture (in a manner similar to macrophage activation when stimulated with endotoxin) since only IL-6, and not IL-1 or TNF, production is high. Alternatively, proximal tubules may constitutively produce IL-6 *in vivo*, but this has not been well studied.

Stx-1 stimulates inflammatory cytokine production by human proximal tubule cells. The mechanisms by which this occurs appear to vary and depends upon the specific cytokine being studied. In the case of TNF, Stx-1 augments release and mRNA levels by mechanisms that are, at least in part, independent of global inhibition of protein synthesis or cytotoxicity. The evidence for this is twofold: (*1*) Stx-1 can stimulate TNF production under conditions with no detectable cellular injury or blockade of protein synthesis; and (*2*) CHX, at concentrations that mildly to severely inhibited protein synthesis or were cytotoxic, had no effect on proximal tubule cell TNF release or mRNA accumulation. The current study did not investigate the precise

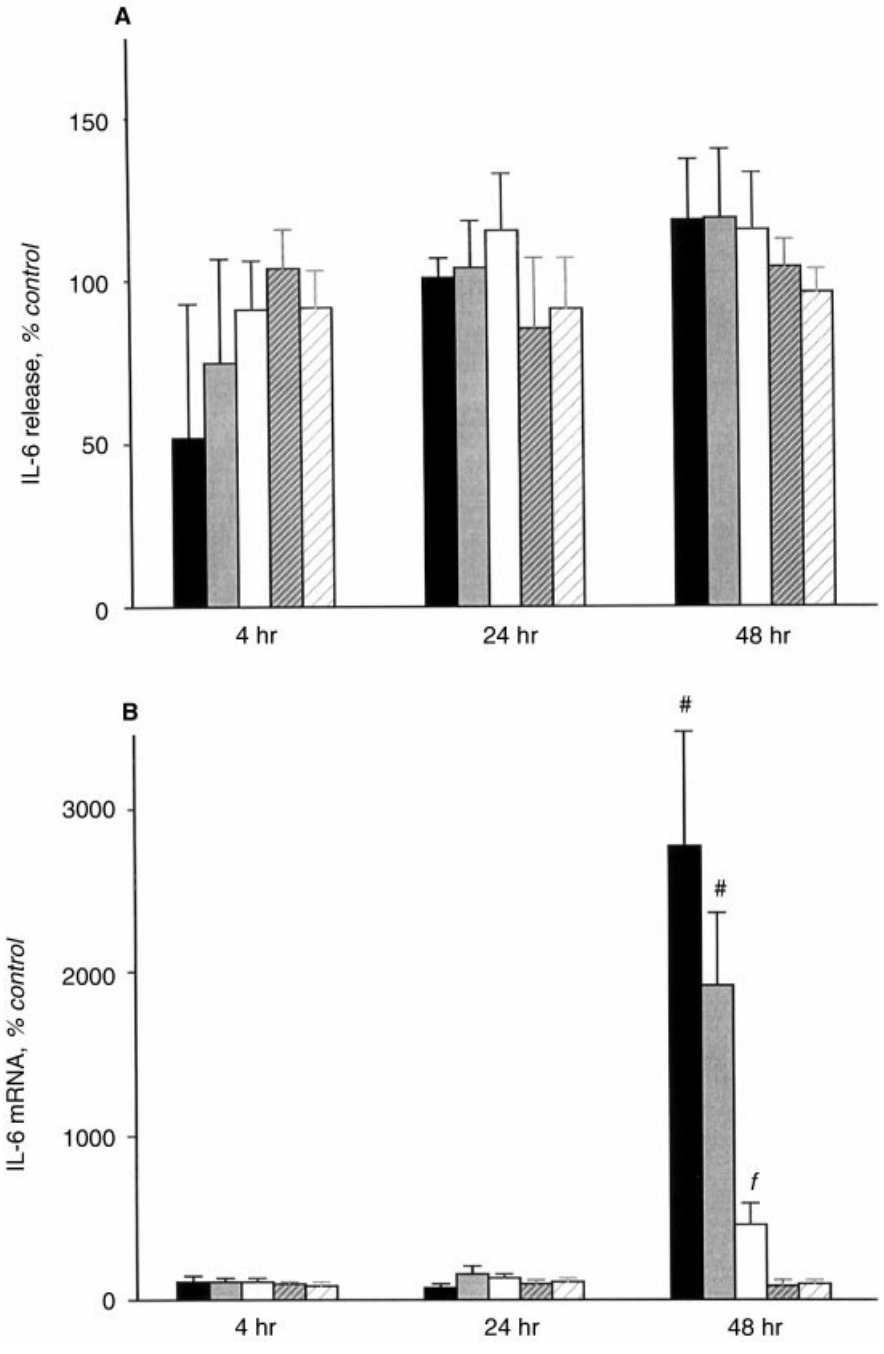

Fig. 11. Effect of cycloheximide on human proximal tubule cell IL-6 release (*A*) and IL-6 mRNA levels (*B*). Results are expressed as percent of control where baseline IL-6 release was 9566 ± 580 ng IL-6/mg total cell protein/24 hr and baseline IL-6 mRNA levels were 6.64 ± 2.75 fg IL-6 cDNA/ng β-actin cDNA. $N = 6$ per IL-6 release data point and $N = 3$ per IL-6 mRNA data point. $^{f}P < 0.01$, $^{\#}P < 0.001$; both versus control. Symbols are: (■) 100 μM; (▨) 50 μM; (□) 10 μM; (▨) 1 μM; (▨) 0.1 μM.

mechanism(s) by which Stx-1 regulates TNF production, as this was beyond the scope of these initial investigations. Nonetheless, there are several considerations relevant to possible mechanisms that are of interest. First, CHX has been reported to have no effect on TNF mRNA levels in resting human monocytes, but to increase TNF gene transcription and reduce mRNA degradation in activated monocytes (so-called "superinduction" of mRNA) [32]. This suggests that the effect of protein synthesis inhibitors on cytokine production may depend, in part, upon factors modulating intrinsic cell responsiveness. For example, CHX up-regulation of TNF in macrophages may depend upon the ability to induce nuclear factor-κB (NF-κB) [33]. Secondly, protein synthesis inhibitors have been described to differentially regulate intracellular signaling processes. For example, anisomycin and CHX can act as nuclear signaling agonists to stimulate pp35/pp15, while emitine and puromycin do not [34]. This suggests that agents labeled "protein synthesis inhibitors" may have cellular effects independent of their protein synthesis' inhibitory

activity and that are specific to a given type or class of agents. Third, protein synthesis inhibitors have been demonstrated to increase mRNA and protein levels for specific proteins while reducing overall protein synthesis. For example, CHX increases superoxide dismutase, c-*fos*, c-jun, and *bcl-2* mRNAs and protein levels in neuronal cells while reducing overall protein synthesis by up to 40% [35]. It is evident from these considerations that Stx-1 stimulation of TNF production may be quite complex and could potentially involve mechanisms both dependent and independent of protein synthesis inhibition.

Stx-1 increased IL-1 release and mRNA accumulation by human proximal tubule cells. This effect occurred at toxin concentrations ranging from not detectable to marked inhibition of protein synthesis. In contrast to its action on TNF, CHX had almost identical effects as Stx-1 on proximal tubule cell IL-1 production. Thus, Stx-1 stimulation of IL-1 production appears to be a nonspecific effect resulting from mechanisms also activated by CHX. These mechanisms could be quite complex and could relate to either global inhibition of protein synthesis and/or to other effects of both agents. It is relevant to note that even the superinduction of IL-1 mRNA transcripts observed in this study could occur by a variety of mechanisms. For example, cytokine mRNA induction by protein synthesis inhibitors has been variously ascribed to stimulation of gene transcription and/or stabilization of mRNA. Induction of IL-1 mRNA by CHX has also been found to be due to enhanced processing of unstable IL-1 precursor transcripts into mature mRNA [36]. Detailed studies will be needed to sort out these possibilities.

Both Stx-1 and CHX stimulated IL-6 mRNA accumulation, while neither agent modified IL-6 release. Stx-1 and CHX only stimulated IL-6 mRNA accumulation at concentrations that substantially inhibited protein synthesis, and this may have prevented an increase in IL-6 translation. Induction of IL-6 mRNA, as discussed above for the other cytokines, could have occurred by a variety of mechanisms, however, it is tempting to speculate that IL-6 mRNA accumulation was a nonspecific effect associated with severe cell injury.

LPS had no effect on inflammatory cytokine production by human proximal tubule cells under baseline conditions. This finding is supported by earlier work demonstrating either no detectable or a small effect of LPS on proximal tubule TNF or IL-6 production [30, 31, 37]. LPS also did not alter Stx-1 modulation of IL-6 or TNF production; however, the endotoxin did enhance Stx-1 stimulated IL-1 release and mRNA accumulation. LPS augmentation of Stx-1-stimulated IL-1 production is of particular interest in light of previous studies demonstrating that IL-1, but not TNF or IL-6, increases proximal tubule sensitivity to the cytotoxic effect of Stx-1 [11]. It will clearly be of interest to determine whether LPS and Stx-1 stimulated production of IL-1 by proximal tubules has an autocrine effect on protein synthesis and/or cell survival.

What is the significance of Stx-1 and/or LPS elaboration of proximal tubule cytokine production in D+HUS? Although this question has not yet been answered, a large number of possibilities exist. As alluded to earlier, these include multiple effects of cytokines on various renal cell types, including increases in: (*1*) Stx-1 cytotoxicity [6, 14]; (*2*) vasoactive factor release (such as endothelin-1 secretion from glomerular endothelial cells [38]); (*3*) oxygen radical formation [39]; (*4*) tissue factor release [40]; (*5*) direct cytokine cytolytic effects [41]; (*6*) fibrin accumulation [42]; and (*7*) numerous other effects [43]. The current study has taken the first steps, by demonstrating that proximal tubule cells are potently stimulated by Stx-1 to produce these inflammatory cytokines. The challenge now remains to dissect out the role that these and possibly other cytokines play in the pathogenesis of renal injury in D+HUS.

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APPENDIX

Abbreviations used in this article are: CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; D+HUS, diarrhea hemolytic uremic syndrome; DMDM:F12, Dulbecco's modified Eagle's media:Hamm's F12; EIA, enzyme immunometric assay; GB_3, galactose-α-1,3, galactose-B-1,4, glucose-ceramide; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PBS, phosphate buffered saline; Stx-1, Shiga toxin-1; TCA, tricarboxylic acid; TNF, tumor necrosis factor.

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