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Endocytosis of light chains induces cytokines through activation of NF-κB in human proximal tubule cells

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Background. In proteinuric states increased cytokine production through endocytosis of filtered proteins by proximal tubule cells (PTCs) has been proposed as a major mechanism mediating tubulointerstitial injury and progressive kidney disease. We studied the effects of six different light chains (LCs) on the production of cytokines in cultured human PTCs.

Methods. LCs were isolated and purified from the urine of patients with myeloma and human PTCs were exposed to either LC or human serum albumin (HSA) for up to 24 hours. LC endocytosis was monitored by immunocytochemistry. Cytokines were determined by enzyme-linked immunosorbent assay (ELISA) in the supernatants and activation of nuclear factor-kappa B (NF-κB) was detected by electrophoretic mobility shift assays (EMSA) and immunocytochemistry.

Results. Endocytosis of LCs induced the release of interleukins (IL) IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1); however, there was considerable variability among the six different LCs. In contrast, HSA had no effect on cytokine production even at very high concentrations. Removal of LC-containing media resulted in cessation of IL-6 release. LC-induced cytokine release was associated with nuclear translocation of NF-κB subunits p50 and p65, as demonstrated by both EMSA and immunocytochemistry. Inhibitors of NF-κB, aspirin and pyrrolidineditiocarbamate (PDTC) markedly suppressed LC-induced cytokine production.

Conclusion. LC endocytosis leads to production of inflammatory cytokines through activation of NF-κB. This may be an important mechanism of chronic tubulointerstitial inflammation process commonly seen in multiple myeloma. These findings also point out a potential role by filterable low-molecular-weight proteins, like LCs, in PTC injury during all proteinuric diseases.

Key words: interleukin-6, monocyte chemoattractant protein-1, proteinuria, progressive kidney disease, human serum albumin, myeloma.

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Recent studies have focused on protein overloading of proximal tubular epithelia and the resultant cytokine production as an important mechanism of interstitial inflammation responsible for progression of chronic renal insufficiency in proteinuric diseases [1–8]. In experimental studies, increased endocytosis of filtered proteins by proximal tubule cells (PTCs) has been shown to induce proinflammatory and inflammatory cytokines by PTCs, possibly mediating tubulointerstitial injury [3, 4, 7]. This mechanism has emerged as an important contributor of interstitial inflammation and progression of renal disease in proteinuric states.

Multiple myeloma is a B-cell neoplasm characterized by bone marrow infiltration with malignant plasma cells, which synthesize and secrete monoclonal immunoglobulin fragments [9]. It is the sixth most common malignancy, affecting approximately 14,000 new individuals per year in the United States, and remains incurable despite all available therapies [10]. Renal failure is a common accompaniment of multiple myeloma and is identified in up to 50% of patients at presentation. This complication carries a worse prognosis and low response rate to chemotherapy [11, 12]. Excessive production of light chains (LCs) in multiple myeloma and the associated LC proteinuria often cause various renal disorders. These disorders include Fanconi's syndrome, reflecting PTC toxicity [13, 14], concentration and acidification defects, indicative of distal tubule cell toxicity [15] and cast nephropathy associated with binding of LCs to Tamm-Horsfall proteins in the distal tubule lumen [16, 17]. The most common histologic lesion is cast nephropathy, often termed "myeloma kidney" [18]. LC cast formation in distal tubules in association with Tamm-Horsfall proteins leads to chronic renal disease, but the precise mechanisms of chronic tubulointerstitial injury and the role of PTCs in this process have not been fully elucidated.

In multiple myeloma excessive production and luminal delivery of LCs to PTCs causes increased endocytosis of LCs via the endocytic receptors cubilin and megalin and

result in eventual degradation within the lysosomes [19, 20]. Therefore, excessive endocytosis of LC is likely to strain the endocytic-catabolic apparatus, possibly resulting in release of endosomal/lysosomal contents into cytosol and triggering an inflammatory response, as has been observed with albumin exposed cells.

In the present study, we evaluated whether cell-traffic overloading by LCs might induce inflammatory cytokines implicated in progression of kidney disease, such as interleukin (IL)-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), regulated-on-activation-normal T-cell-expressed-and secreted (RANTES), tumor necrosis factor (TNF)-α and transforming growth factor-β1 (TGF-β1). We characterized the cytokine responses in cultured PTCs to six different LCs isolated from the urine of myeloma patients with predominantly tubulointerstitial disease.

METHODS

Isolation and purification of myeloma LCs

Light chains were isolated and purified from the urine of multiple myeloma patients by slight modification of the method previously reported from our laboratory [21]. LCs were precipitated from urine with ammonium sulfate (55 to 90% saturation-determined empirically), extensively dialyzed against distilled water and lyophilized. The precipitated LCs were purified by dissolving the lyophilized desalted crude protein in buffer at pH 6.0, followed by chromatography on carboxymethyl-Sephadex (C-50; Pharmacia, Piscataway, NJ, USA). Under these conditions, the LCs were bound to the column, whereas the contaminants were not. Bound LC was eluted with 0.6 mol/L of NaCl, redialyzed against distilled water, and lyophilized. The purity of LCs was confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis [22] and the immunologic identity reported from the clinical laboratory was confirmed by Western blotting using goat anti-human κ and λ antibodies.

These LC preparations were tested for endotoxin using the chromogenic Limulus amebocyte lysate (LAL) test (Charles River Labs, Charleston, SC, USA). Endotoxin has previously been shown to induce MCP-1 gene expression through activation of nuclear factor-κB (NF-κB) in rat tubular epithelial cells at 1 to 10 μg/mL concentrations [23] corresponding to approximately 3000 to 30,000 EU according to the manufacturer's specifications. We found that all six LCs used in these experiments were essentially endotoxin free (0.1 to 3.7 EU/mg LC protein). The two human serum albumin (HSA) preparations, which were used as negative controls, had 3.1 and 8.5 EU/mg endotoxin. In additional experiments, no effect of endotoxin was found up to 7500 EU/mL on induction of IL-6 in our cell preparations, nor did the addition of endotoxin to LC in these concentration ranges have any additive or potentiating effect on LC-induced cytokine production.

Light chains were collected from patients with minimal or no albuminuria. Thus, the LCs studied here represent tubulopathic LCs with no clinical evidence of glomerular involvement. The serum creatinine concentrations of these patients ranged from 1.6 to 3.3 mg/dL. Many patients showed an improvement in renal function after they were started on chemotherapy and supportive treatment. Kidney biopsies were not performed in any of the patients.

Because the LCs were collected over five years from patients in several different hospitals affiliated with our program, a detailed follow-up of the eventual clinical outcomes was not possible.

The effects of six species of LCs, four κ and two λ chains, were evaluated on cytokine secretion. These six LCs were the same LCs studied in our previous report [21]. Two LCs (LC-1, a κ-LC and LC-2, a λ-LC) that demonstrated significant effects on IL-6 secretion were selected for additional studies.

Cell cultures

Initially the effects of LCs on cytokine production in primary cultures of human PTCs (Clontech, Palo Alto, CA, USA) were evaluated. After confirming that cytokine repertoire was identical in both the primary cultures and SV40 immortalized human kidney PTCs, all experiments were conducted using SV40 PTCs and only data obtained in this cell line are presented here. Grown as a monolayer, these cells showed marker brush-border enzymes and had biochemical and morphological characteristics similar to other widely used PTC lines, including LLC-PK1, OK, HK-2, and human PTCs in stable culture [24]. Brush border enzymes and phloridzin-inhibitable glucose transport were similar to other established PTC models. This transformed cell line's response to inflammatory stimuli was extensively compared with parental PTCs and was found to be similar [25]. Cells were routinely grown in DRM-23E [24] medium supplemented with 0.5% (vol:vol) fetal bovine serum in T-75 flasks (Falcon, Becton Dickinson Labware, NJ, USA), in the presence of 5% CO_2 at 37°C.

Tracking LC endocytosis by indirect immunofluorescence

Endocytosis of LC was tested by indirect immunofluorescence [26] using fluorescein-isothiocyanate conjugated (FITC) secondary antibodies against anti-LC antibodies. For this purpose, cells were grown on glass cover slips and then permitted to undergo endocytosis of unlabeled LC. Control slides were exposed to LC-1 at 0°C to stop endocytosis. Cells were allowed to endocytose LC-1 (200 μmol/L) in the medium and then cells were fixed in 2% para-formaldehyde (vol:vol) in phosphate-buffered saline (PBS) made up of 20 mmol/L sodium phosphate, 150 mmol/L NaCl, and 2 mmol/L $MgCl_2$ at

pH 7.4. After the cells were washed with PBS, they were permeabilized with 2 mL of 0.01% (vol:vol) Triton X-100 in PBS buffer for five minutes at 10°C and stained with anti-human LC antibody followed by FITC conjugated secondary antibody. Endocytosis experiments were done using an immunofluorescence microscope and a digitized photomicrography system (Version 3.6; Metamorph Universal Imaging, West Chester, PA, USA).

Evaluation of the effects of LCs and increasing concentrations of HSA on cytokine production in PTC

When SV40 PTCs formed a confluent monolayer in T-75 flask, they were trypsinized and transferred to 24-well tissue culture plates (Costar, Corning Incorporated, NY, USA). After reaching confluency in 24-well plates, cells were first exposed to 400 μmol/L concentrations of six LCs (LC-1 to LC-6) and HSA for 24 hours. At the end of exposure period, supernatants were harvested and stored at −70°C for cytokine assays. Results from initial screening experiments were used in planning detailed studies. The initial experiments showed significant increases in IL-6, IL-8 and MCP-1, but no significant changes in IL-1, TGF-β1, RANTES and TNF-α. Because of the unique associations between myeloma and IL-6, we focused on this cytokine. For these studies LC-1 (κ) and LC-2 (λ) were selected as they had the most potent stimulatory effects on cytokine production.

Detailed time-course and dose-response studies for IL-6 production were conducted using LC-1. Confluent cell monolayers in 24-well plates were exposed to varying concentrations (0, 25, 50, 100, 200, 400 μmol/L) of LC-1 for varying time intervals (2, 6, 20, 48 hours). To verify the LC effect, after incubation with LC-1, LC containing medium was removed and replaced with LC free medium, and IL-6 levels were tested in supernatants at one, two and four hours. Supernatants were harvested and stored at −70°C at each step for cytokine assays.

Light chain-1 and HSA were dissolved in regular cell culture medium. Confluent monolayers of cells were exposed to LC-1 (50 and 100 μmol/L) and HSA (50, 100 μmol/L) for 6 and 24 hours in 24-well plates. Supernatants were harvested from each well for cytokine assays. HSA was tested at high concentrations (10 and 30 mg/mL, approximately 160 and 500 μmol/L) to compare cytokine responses with LCs.

Interleukin-6, IL-8, MCP-1, RANTES, TGF-β1, TNF-α and IL-1 were measured in the supernatants using commercial human ELISA kits (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The sensitivity of the ELISA is less than 0.7 pg/mL, 10 pg/mL, 5 pg/mL, 8 pg/mL, 7 pg/mL, 4.4 pg/mL and 1 pg/mL for IL-6, IL-8, MCP-1 and RANTES, TGF-β1, TNF-α and IL-1 assays, respectively. Cytokine concentrations in the unknown samples were determined by interpolation into a standard curve developed with known amounts of recombinant human cytokines. Experiments were conducted in triplicate using 96-well micro plates and the results were read in a micro plate reader. Cells were trypsinized and counted to express the amount of cytokine as ng per 10^6 cells.

Maneuvers that interfere with LC endocytosis

For this purpose, two different strategies were used. The first maneuver was pretreatment and treatment with bafilomycin A, which alkalinizes endocytic vesicles by specifically inhibiting vesicular H^+-adenosinetriphosphatase [19]. After a one hour pretreatment with bafilomycin A (1 μmol/L) at 37°C, cells were exposed to LC-1 (50 μmol/L) + bafilomycin A (1 μmol/L) for six hours, then supernatants were harvested and stored at –70°C for cytokine assays. Second, LC-1 exposure was repeated in medium made hypertonic with 0.25 mol/L sucrose (ICN, Costa Mesa, CA, USA). Hypertonic medium is known to interfere with clathrin lattice formation and is used as a maneuver to disrupt the clathrin-coated endocytic pathway but not fluid-phase endocytosis [19]. Cells were incubated in hypertonic medium for one hour at 37°C. After pretreatment with hypertonic sucrose (0.25 mol/L), confluent monolayers of cells were exposed to 50 μmol/L of LC-1 for six hours in the continued presence of 0.25 mol/L sucrose. At the end of the exposure, supernatants were harvested and stored for ELISA at −70°C.

Determination of NF-κB activation

To determine whether LC-induced cytokine production is through activation of the NF-κB, nuclear translocation of NF-κB was evaluated by both immunocytochemistry and EMSA [3, 27].

Immunocytochemistry. Cytoplasmic and nuclear localization of NF-κB p65 and p50 subunits were determined by immunofluorescence according to methods described previously [28], modified slightly according to the protocol provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), the source for the antibodies. SV40 cells were grown on sterile glass cover slips, then placed in 24-well tissue culture plates for overnight at 37°C and exposed to LC-1, HSA, 100 μmol/L each, and standard media for one hour. At the end of exposure, cells were fixed using methanol for five minutes at −10°C and incubated with 10% normal goat serum for 30 minutes to eliminate non-specific binding of IgG, and then washed with PBS. Anti-NF-κB p65 and p50 were added (1:100) at room temperature for 60 minutes. After washing with PBS, cells were incubated with secondary fluorescein-conjugated antibody (1:100) for 45 minutes and then were mounted by 90% glycerol. Immunofluorescent microscope and a digitized photomicrography system (Metamorph Universal Imaging, Version 3.6) were used to evaluate the cells.

Electrophoretic mobility shift assay (EMSA). Nuclear

extracts from cells were prepared as described by Zoja et al [3]. Confluent monolayers of cells were incubated with 100 μmol/L LC-1, LC-2 and HSA for six hours. After washing with PBS, cells were harvested and resuspended in 2 mL hypotonic buffer [10 mmol/L Hepes, pH 7.6, 15 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1.0 mmol/L dithiothreitol (DTT), and 0.2% Nonidet P-40]. The suspension was centrifuged at 650 × *g* after incubating for five minutes on ice and the pellet was then washed once with the same buffer. The resulting nuclear pellet was resuspended in 300 μL buffer [25 mmol/L Hepes, 50 mmol/L KCl, 0.1 mmol/L EDTA, pH 8.0, 1.0 mM DTT, 10% (vol:vol) glycerol] and 0.4 mol/L NaCl. This suspension was incubated for 45 to 60 minutes at 4°C and centrifuged at 18,000 × *g* for 10 minutes. The supernatant, which is the nuclear extract, was divided into aliquots and stored at −70°C until use.

The oligonucleotide used as a probe for EMSA was a 42-bp double-stranded construct (wild-type) 5′-TTGT TACAAGGGGACTTTCCGCTGGGGACTTTCCAG GGAGGC-3′ containing two tandemly repeated NF-κB binding sites (underlined) were obtained from Dr. Vancurova (New Hyde Park, NY, USA). Mutant oligonucleotide used for competition studies was 5′-TTGTTACAATCT CACTTTCCGCTTCTCACTTTCCAGGGAGGC-3′. End labeling was accomplished by treatment with T4 kinase in the presence of ^{32}P ATP, and the labeled oligonucleotide was purified on a Sephadex G-25 column, as described [29]. Briefly, nuclear protein extracts (5 μg protein, 10^6 cell equivalent, in 5 to 7 μL) prepared from PTCs were incubated (20 min at room temperature) with 5 to 10 fmol of radiolabeled oligonucleotide (~70,000 cpm) in 20 μL of binding buffer (20 mmol/L Tris-Cl, pH 7.5, 150 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% NP-40, 6% glycerol) supplemented with 20 μg acetylated BSA and 2 μg poly (dI-dC). For competition experiments, binding reactions were performed in the presence of 30 molar excess of unlabeled (wild-type) oligonucleotide before adding the ^{32}P labeled probe. For supershift experiments, 1 μg of specific polyclonal antibody against p50, p65 and c-rel were added and incubated 30 minutes at room temperature before introducing the ^{32}P-labeled oligonucleotide. The resulting complexes were resolved on 5% non-denaturing polyacrylamide gels that had been pre-run at 100 V for 30 minutes in 0.5 × TBE buffer. Electrophoresis was conducted at 180 V for 2.5 hours. After electrophoresis, gels were transferred to Whatman DE-81 paper, dried and exposed to autoradiographic film (Kodak Biomax MS; Eastman Kodak, Rochester, NY, USA) with an intensifier screen at −80°C. Each gel was exposed to the film for different lengths of time to generate series of images with different intensities.

Effects of NF-κB inhibitors aspirin and PDTC on cytokine production

To confirm the role of NF-κB, the effects of NF-κB inhibitors aspirin (20 mmol/L) [30] and pyrrolidineditiocarbamate (PDTC; 12.5μmol/L) [31] for one hour prior to and during six hours of incubation with LC-1 (50 μmol/L) were studied on cytokine production. At the end of the incubation, supernatants were harvested and stored for cytokine assays. Aspirin and PDTC were dissolved in regular media and freshly prepared each time before the experiments. The concentration of aspirin (20 mmol/L) and PDTC (12.5 μmol/L) used were determined from pilot studies.

All antibodies and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Statistical analysis

Results were expressed as mean ± SEM. Multiple comparisons were made by analysis of variance (ANOVA) and Bonferroni's multiple-comparison tests. Statistical analyses, curve fitting, and calculations were done using GraphPad Prism, version 3 for Windows NT (1999, GraphPad Software Inc, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

RESULTS

LC endocytosis by indirect immunofluorescence

Light chain endocytosis was confirmed during these experiments by immunocytochemistry before proceeding with cytokine assays. After a four hour exposure there was brisk endocytosis of LC by PTCs with vacuolar localization in the cytoplasm (Fig. 1). We had demonstrated previously that LCs undergo receptor-mediated endocytosis by radioisotope and flow cytometry via binding to cubilin in cultured PTCs [19, 20]. Our work also showed that LC endocytosis occurs through the clathrin-coated pathway and results in significant degradation.

Effects of tubulopathic LCs on IL-6 production

Focusing our studies on IL-6 responses in view of its unique association with multiple myeloma, the effects of six different LCs on the production of IL-6 in SV40 PTCs were evaluated. All LCs stimulated production of IL-6 (3- to 15-fold, compared to control) while HSA had no significant effect (Fig. 2). There was considerable variability among the LCs tested here. Two LCs [LC-1 (κ) and LC-2 (λ)] had the most marked effect on production of IL-6. The IL-6 response to LC-1 was time- and dose-dependent (Fig. 3). The maximum effect of LC-1 was seen within six hours of exposure and persisted for more than 48 hours, the longest time interval tested in these studies (Fig. 3A). The half-maximal effect was

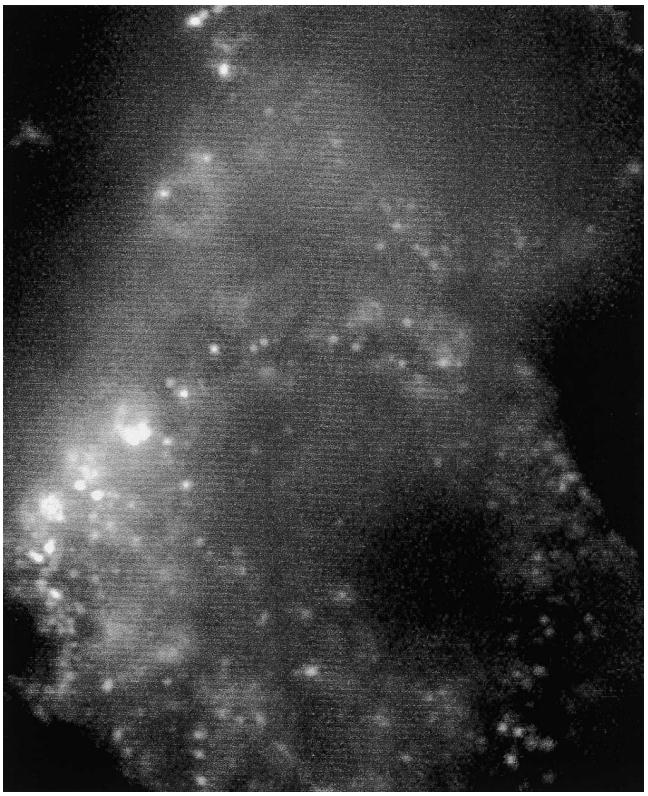

Fig. 1. Tracking light chain-1 (LC-1) endocytosis by immunocytochemistry in proximal tubule cells. LC-1 is localized in the cytosol of two adjacent cells in vacuolar distribution (×100, oil immersion; details are in the **Methods** section).

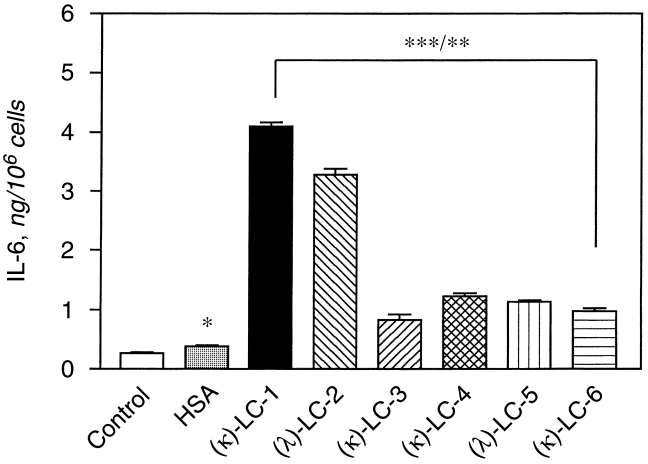

Fig. 2. Effects of six different light chains (LCs) and human serum albumin (HSA) on interleukin-6 (IL-6) production. All LCs stimulated the production of IL-6 in 24 hours (***$P < 0.001$ vs. control, **$P < 0.001$ vs. HSA), while HSA had no effect (*$P > 0.05$ vs. control). Results are presented as mean ± SEM.

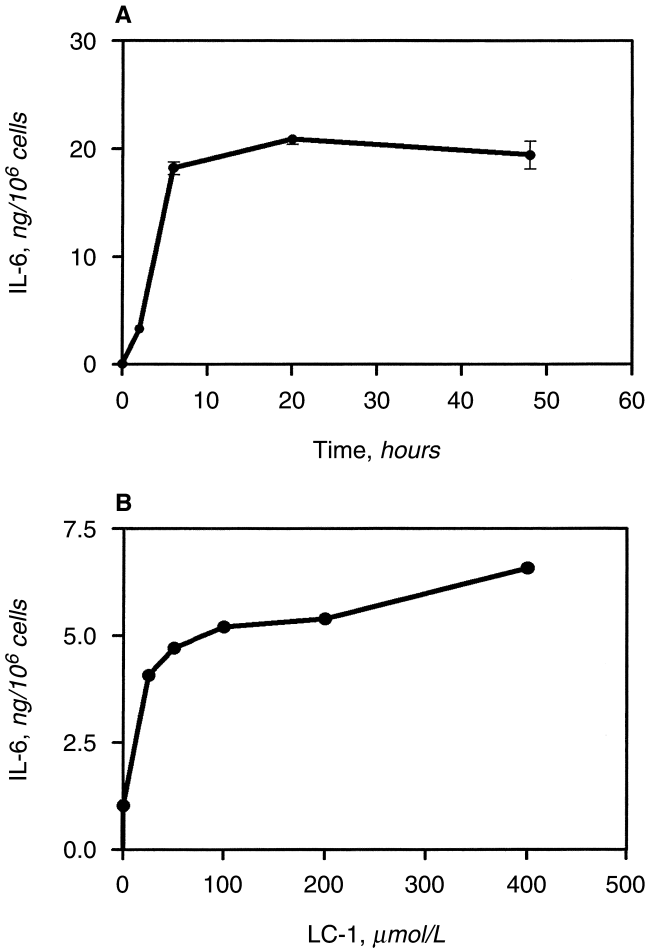

Fig. 3. Time-course and dose-response of IL-6 induction by LC-1. (*A*) Time-course study showed that the maximal effect of LC-1, 400 μmol/L, on IL-6 production was seen at six hours and this effect was sustained over 48 hours; longer time intervals were not studied. (*B*) Dose-response experiment showed that half-maximal stimulatory dose of LC-1 was ~20 μmol/L at the end of a 24-hour exposure. Each point represents the mean of triplicate measurements.

observed at approximately 20 μmol/L concentration of LC-1 (Fig. 3B). These concentrations are well within the range that can be achieved in the proximal tubule fluid of a patient with multiple myeloma. Free LC concentration in the serum of myeloma patients can be more than 1000-fold of normals; for example, free κ-LCs ranged from 4.2 to 3514 mg/L and free λ-LCs from 1.1 to 7770 mg/L in a report by Solling et al [32], yielding theoretical proximal tubule fluid concentrations up to approximately 300 μmol/L, and exceeding these levels as tubular fluid is reabsorbed along the proximal tubule. Furthermore, free LC excretion in the urine of myeloma patients can be as high as 32 g/day [33].

To confirm the role of LC in the induction of IL-6, LC containing medium was removed and the same cells incubated with LC free medium; the continued presence of LC was required for a continued release of IL-6 into medium (Fig. 4). We also looked for IL-6 in LC containing medium before all experiments and did not detect measurable quantities of IL-6, indicating that IL-6 con-

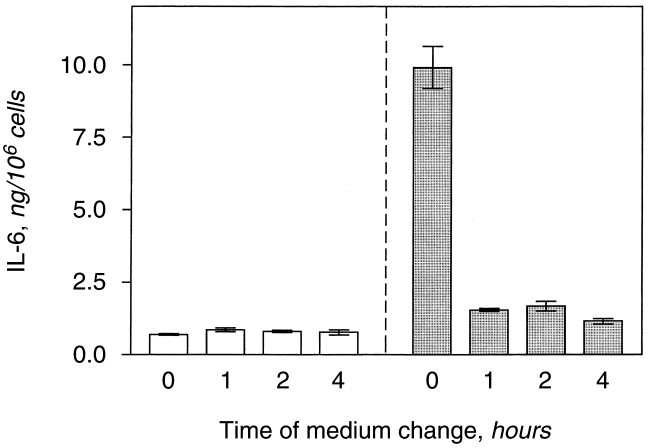

Fig. 4. IL-6 production requires the continuous presence of LC-1. Cells were incubated in medium without (□) or with (■) LC-1 for 24 hours. Upon replacement with fresh medium (time 0) containing no LC-1, IL-6 production promptly ceased and by 1 hour was not different from time-matched controls.

tamination does not occur during purification of LC from the urine of the patients.

Effects of LC-1 and HSA on IL-6, IL-8 and MCP-1 production

Light chain-1 (1.5 and 3 mg/mL; ~50 and 100 μmol/L) stimulated production of IL-6 at six and 24 hours of exposure, while HSA had no effect even at high concentrations (10 and 30 mg/mL, ~160 and 500 μmol/L) at the same exposure intervals (Fig. 5).

The effects of increasing concentrations of HSA and LC-1 (50, 100 μmol/L) on IL-8 and MCP-1 production by PTCs are shown in Figures 6 and 7. After 6 and 24 hours of exposure to LC-1 there were increased productions of IL-8 (Fig. 6) and MCP-1 (Fig. 7). In contrast, exposure to HSA, even at a 30 mg/mL concentration (~500 μmol/L concentration) had no effect on IL-8 and MCP-1 production. None of the LCs studied here had stimulatory effects on the production of TGF-β1, IL-1, TNF-α or RANTES (data not shown).

Effects of maneuvers that interfere with LC endocytosis on IL-6, IL-8 and MCP-1 production

Two different maneuvers that interfere with LC endocytosis resulted in inhibition of the cytokine response induced by LC-1 (Fig. 8). First, alkalinization of endosomal contents by bafilomycin A (1 μmol/L), which results in disruption of endosomal fusion and presumably disrupts LC traffic in the cytosol [19], resulted in significant inhibition of the IL-6, IL-8 and MCP-1 (Fig. 8A). Similarly, rendering the medium hypertonic by 0.25 mol/L sucrose [19], an intervention known to disrupt clathrin-coated pathway, reduced the cytokine response to LC-1 by more than 75% (Fig. 8B), attesting to the mechanistic role of LC endocytosis in these responses. In the same

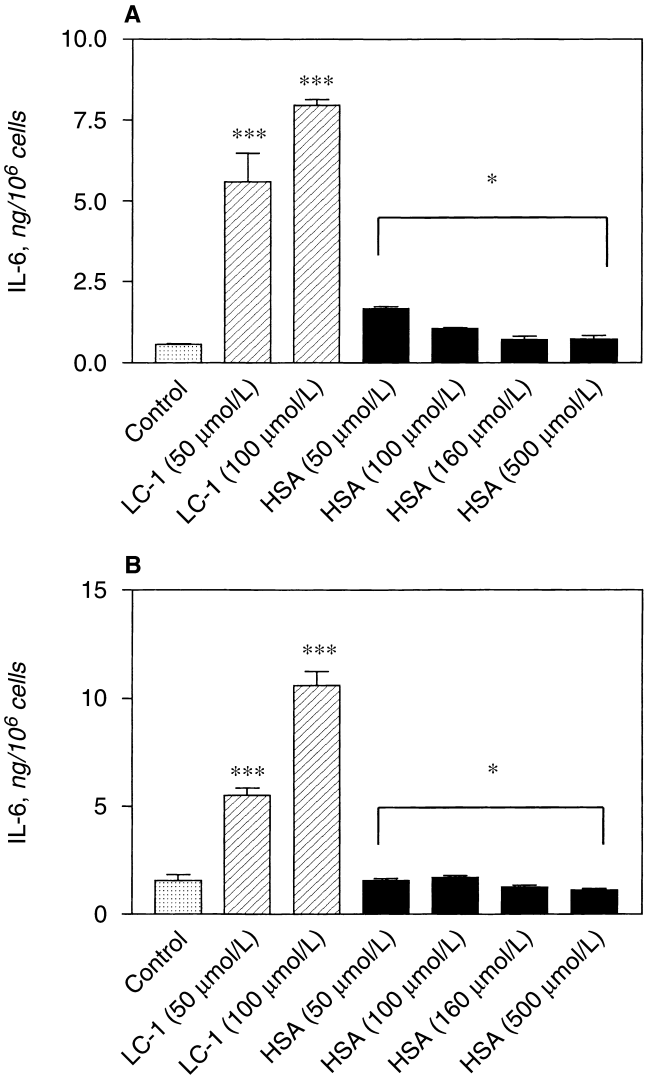

Fig. 5. Effects of LC-1 and HSA on IL-6 production at 6 (*A*) and 24 hours (*B*). Cells were incubated with control medium or medium containing LC-1 (50 and 100 μmol/L) and HSA (50 to 500 μmol/L) for 6 (A) and 24 (B) hours. LC-1 both at 50 and 100 μmol/L significantly stimulated IL-6 production at 6 and 24 hour exposures, while HSA had no effect at any concentrations studied. Results are presented as mean ± SEM (***$P < 0.001$ vs. control, *$P > 0.05$ vs. control).

experiments, bafilomycin A and sucrose on cytokine production without the presence of LC-1 had no effects by themselves on cytokine production in PTCs.

NF-κB translocation by immunocytochemistry and EMSA

Activation of NF-κB in PTCs exposed to LC-1, LC-2 and HSA was probed by both immunocytochemistry (Fig. 9) and EMSA (Fig. 10A). By immunocytochemistry, the p65 subunit of NF-κB was seen in a cytoplasmic distribution in control cells (Fig. 9A). In cells exposed to LC-1 and LC-2, p65 was localized primarily to the nuclei (Fig. 9 B, C). In contrast, there was no discernible nuclear translocation of p65 in HSA-exposed cells (Fig.

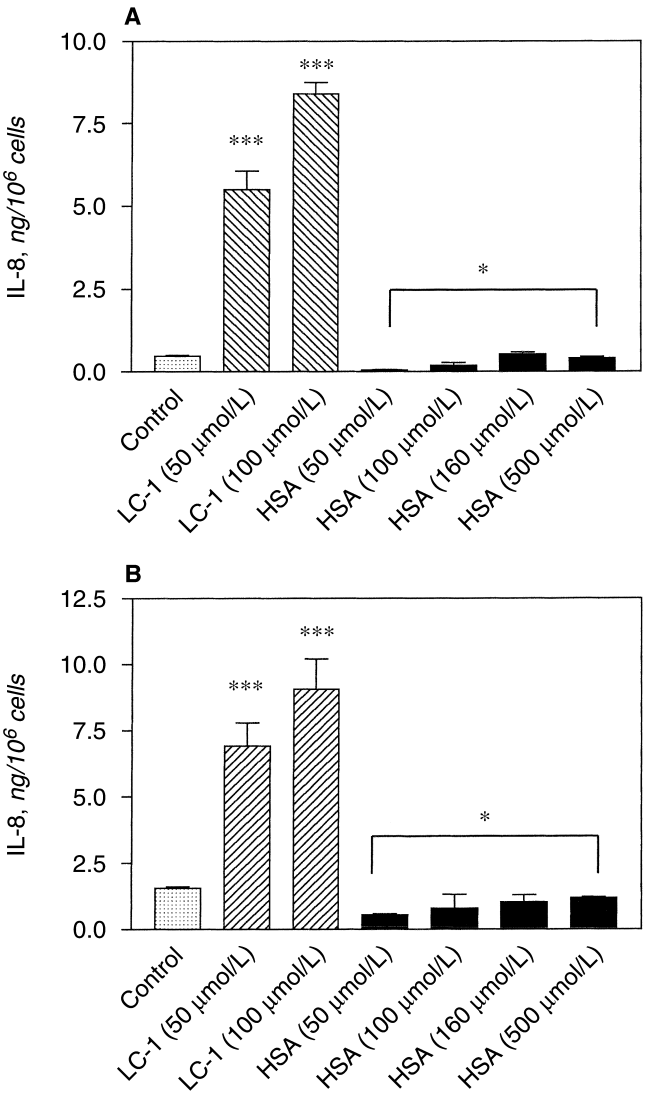

Fig. 6. Effects of LC-1 and HSA on IL-8 production at 6 (*A*) and 24 hours (*B*). Cells were incubated with different concentrations of LC-1 and HSA for 6 (A) and 24 (B) hours. Production of IL-8 was stimulated by LC-1 while HSA had no significant effect even at high concentrations. Results are presented as mean ± SEM (***$P < 0.001$ vs. control, *$P > 0.05$ vs. control).

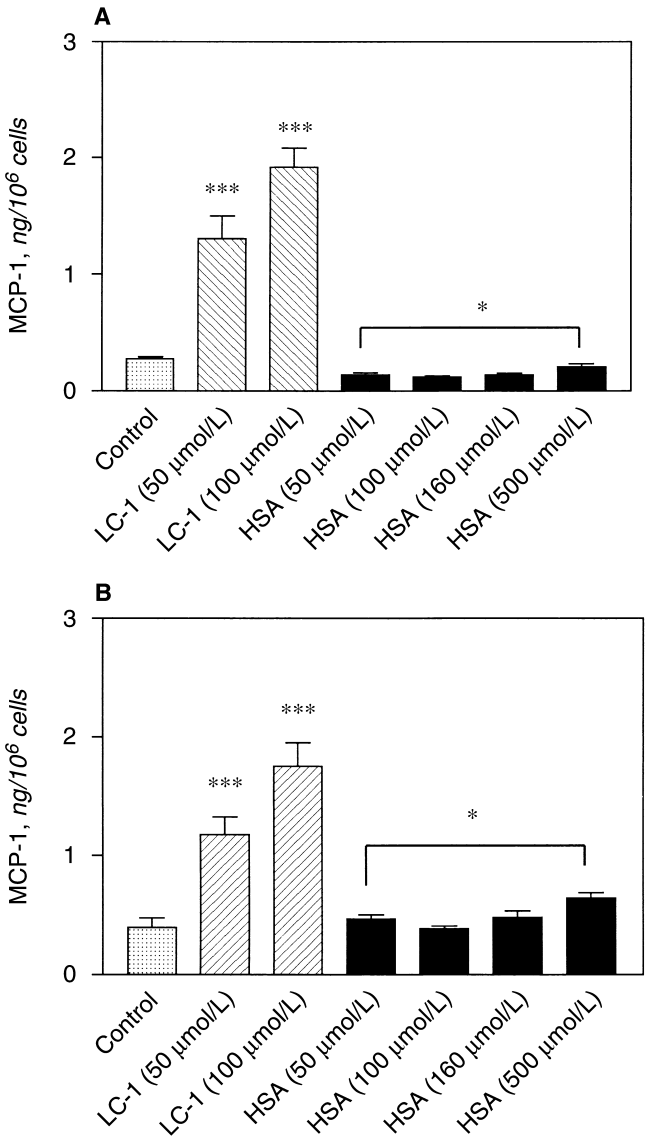

Fig. 7. Effects of LC-1 and HSA on MCP-1 production at 6 (*A*) and 24 hours (*B*). Cells were incubated with different amounts of LC-1 and HSA for 6 (A) and 24 (B) hours. LC-1 significantly stimulated production of MCP-1 while HSA had no effect. Results are presented as mean ± SEM (***$P < 0.001$ vs. control, *$P > 0.05$ vs. control).

9D). Similar responses were observed for p50 distribution (data not shown).

Electrophoretic mobility shift assay (EMSA) experiments with two different LCs (LC-1, a κ and LC-2, a λ) confirmed nuclear translocation of both the p50 and p65 subunits of NF-κB in nuclear extracts (5 and 9.5 μg) obtained from LC exposed cells (Fig. 10A). Again, there appeared to be no distinct response to HSA exposure. To confirm the specificity of the DNA-protein interaction, competition studies were performed with unlabeled oligonucleotides (Fig. 10B). Addition of the 30-fold excess of unlabeled wild-type 42-bp NF-κB oligonucleotide resulted in the complete disappearance of both complexes (Fig. 10B, lane 2), whereas unlabeled mutant oligonucleotide did not affect either band (Fig. 10B, lane 3). To analyze the subunit composition of these two complexes, a supershift assay was performed using polyclonal antibodies against p50, p65 and c-Rel subunits of NF-κB (Fig. 10B, lanes 4, 5 and 6, respectively). The supershift experiment confirmed the presence of both p50 and p65 subunits in the nuclear extracts obtained from LC exposed cells (Fig. 10B).

Effects of NF-κB inhibitors on LC-induced cytokine responses

Introduction of inhibitors of NF-κB, aspirin and PDTC, into the medium nearly eliminated the LC-induced cyto-

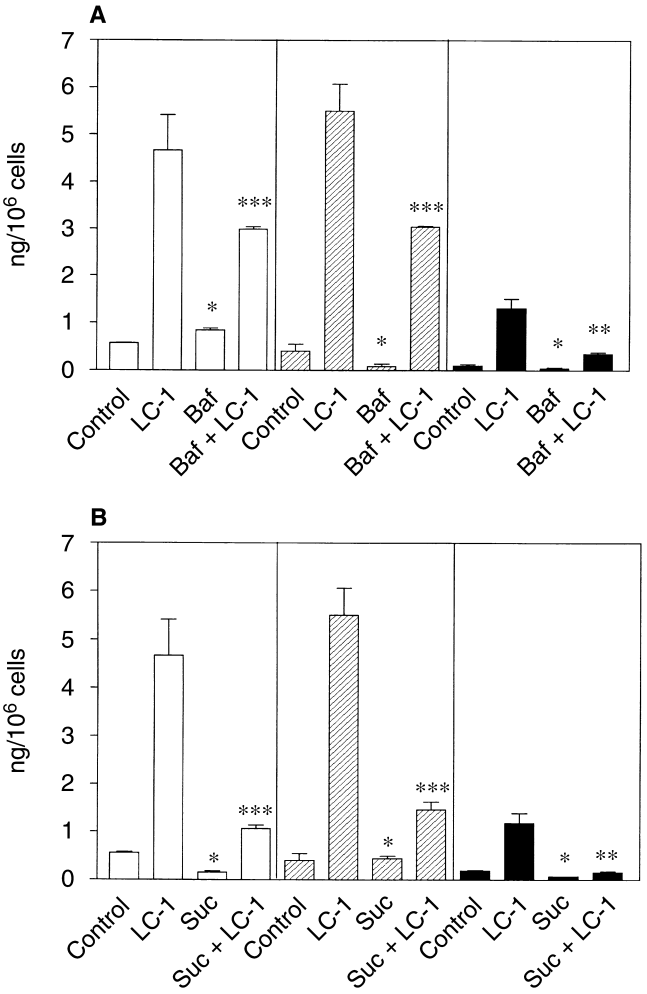

Fig. 8. Effects of bafilomycin A (Baf) and hypertonic sucrose (Suc) on IL-6, IL-8 and MCP-1 production. Bafilomycin A (*A*) and hypertonic sucrose (*B*) inhibited LC-1 induced IL-6 (□), IL-8 (▨), and MCP-1 (■) production. Bafilomycin A alone or sucrose alone had no effect. Results are presented as mean ± SEM (***$P < 0.001$ vs. LC-1, **$P < 0.05$ vs. LC-1, *$P > 0.05$ vs. control).

kine response (Fig. 11), supporting that LC-exposed cytokine induction is mediated through the activation of this transcription factor. In simultaneous experiments, we also tested the effects of the same concentrations of aspirin and PDTC on cytokines production without the presence of LC-1 and found no effect.

DISCUSSION

These studies show that endocytosis of LCs by PTCs can result in induction of pro-inflammatory and inflammatory cytokines. Although our studies focused mostly on IL-6 production, a cytokine uniquely involved with multiple myeloma, they also provide clear evidence of the production of MCP-1 and IL-8 in human PTCs exposed to myeloma LCs. The obvious implication of these observations is that LC-induced cytokine secretion by PTCs may be an important mechanism of the chronic tubulointerstitial inflammation frequently seen in multiple myeloma. Chronic tubulointerstitial nephritis in conjunction with distal tubular casts, formed by binding of LCs to Tamm-Horsfall glycoproteins is the "classical" myeloma kidney [18]. Meticulous investigations by Sanders et al have clarified the pathophysiology of cast formation and have characterized specific binding sequences in Tamm-Horsfall protein with high affinity to "castogenic" LCs [16, 17]. Although these studies offered much insight into the pathogenesis of myeloma kidney, they could not fully link the extensive tubulointerstitial inflammatory cellular infiltration, tubular atrophy and fibrosis to the distal tubule luminal casts. The results of the experiments here reveal a potential role for the most abundant cells in the kidney, the proximal tubular epithelial cells, in the pathogenesis of chronic tubulointerstitial inflammation in patients with LC proteinuria.

Previous studies from our laboratory have demonstrated that myeloma LCs bind to endocytic receptors located in the PTC apical membrane [19, 20, 34]. The recently cloned cubilin is one of these endocytic scavenger receptors, similar to megalin. Radioisotope labeled LC binding and internalization studies and direct binding assays by surface plasmon resonance analysis demonstrated LC interaction with cubilin [20]. That LC binding to apical membranes is followed by internalization and routing to the endosomal-lysosomal degradative pathway was confirmed by antibody inhibition and flow cytometry studies. The present study tracked LC endocytosis by immunocytochemistry and found brisk endocytosis and a vacuolar distribution of LCs in the cytosol of the cells (Fig. 1). This observation suggests that endocytosis and cytokine responses may be causally related. The maneuvers that we had previously demonstrated to inhibit LC endocytosis in PTCs, hypertonicity and bafilomycin A [19], resulted in significant inhibition of cytokine responses (Fig. 8), strengthening the argument that endocytosis is required for cytokine responses. However, the effects of these maneuvers were less than complete, suggesting that mechanisms other than endocytosis, for example, surface binding, also may contribute to these responses.

Similar explanations have been proposed by other investigators who observed that albumin induces cellular stress responses including cytokine release in various PTC cultures, such as, opossum kidney (OK) cells, the porcine cell line LLC-PK1, and human PTCs [3, 6, 7, 35]. These investigators have generally attributed the cytokine responses to "protein overloading" as a result of excessive albumin endocytosis by these cells and have proposed protein overloading as a principal mechanism of progression in chronic renal insufficiency. Although our observations are in general agreement with this concept, there are some significant differences between our findings using LCs and reports in the literature on albu-

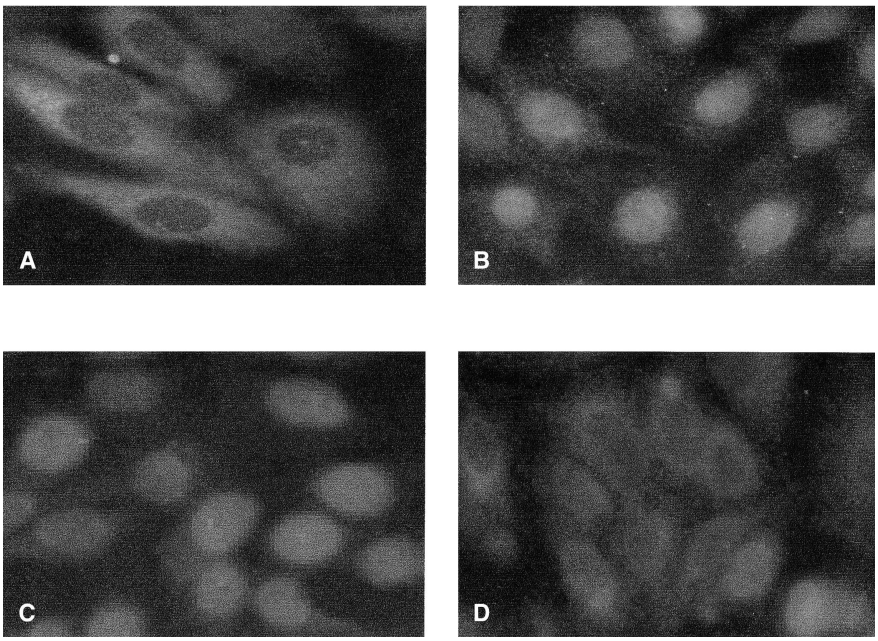

Fig. 9. Localization of NF-κB in PTCs by immunofluorescence microscopy. Cytoplasmic localization of NF-κB (p65 subunit) is seen in unstimulated cells (*A*). After one hour of exposure to LC-1 (*B*) and LC-2 (*C*), fluorescence appears predominantly in the nuclei of the PTCs. In cells exposed to HSA, the immunofluorescence is located predominantly in the cytoplasm (*D*).

min-exposed cells. Most of the experiments have used bovine serum albumin (BSA) with cells derived from other species, such as pig or opossum. In contrast, our experiments used only human kidney cells and our LCs were isolated from humans. Crossing species may have altered immune responses in these in vitro systems. In further and starker contrast, we found that HSA evoked either no or at best minimal cytokine responses, even at very high concentrations, in our human PTCs (Figs. 2, 5 to 7). The quantitative responses seen in our experiments are difficult to compare with those observed with albumin and LLC-PK1 cells, although when normalized to cell numbers, our responses appear several orders of magnitude greater. LCs have a much higher affinity to endocytic receptors than albumin, as we have previously reported in competition experiments [19, 34, 36], and are more likely to be endocytosed in significant quantities than albumin. It is conceivable that in experiments where comparison with a more potent protein, such as LCs, is absent, albumin-evoked cytokine responses may appear significant even if at lesser magnitudes. A relative lack of effect by albumin was recently reported by Bazzi et al, demonstrating that in membranous nephropathy the prognosis of kidney disease correlated more significantly with the quantity of immunoglobulin in the urine than albumin [37]. Nevertheless, many investigators have observed some albumin endocytosis in PTCs [3, 7, 31, 35] and the differences observed here cannot absolve albumin as a potential participant in protein overloading hypothesis.

A significant component of the protein-overloading hypothesis is that excessive endocytosis causes activation of transcription factors triggering nuclear transcription of inflammatory and pro-inflammatory cytokines. The families of nuclear transcription factors, such as NF-κB, that regulate numerous cell injury/inflammation reactions have attracted considerable attention in studies addressing the role of protein overloading in chronic renal disease [3, 6–8, 31, 38]. These transcription factors participate in various injury responses including interactions with chaperone proteins, heat shock proteins, cytokines, apoptotic pathways and oxidative stress [39]. The NF-κB family includes the subunits p50, p52, p65, etc., and an inhibitory IκB subunit [39–41]. These components are constitutively present in the cytoplasm and form dimeric complexes. All known inflammatory stimuli, including many viruses, involve interactions with cell surface receptors initiating a phosphatase cascade that ends with a kinase (known as IκB kinase or IKK), which phosphorylates IκB [39]. This permits serial insertion of a chain of ubiquitin proteins on IκB, which induces the release of IκB, freeing the NF-κB subunits to be translocated to the nucleus and bind to defined promoter sequences on multiple inflammatory genes, releasing diverse repertoire of inflammatory mediators [40, 41]. Our results show that LC endocytosis triggers nuclear translocation of NF-κB (Figs. 9 and 10), suggesting that the cytokine responses occur through activation of these transcription factors. Although NF-κB activation has been attributed to "protein overloading" of cytosolic traffic, the precise mechanisms and the patterns of signaling pathways, such as mitogen-activated protein kinases, involved in this response remain to be characterized.

That the NF-κB inhibitors aspirin and PDTC amelio-

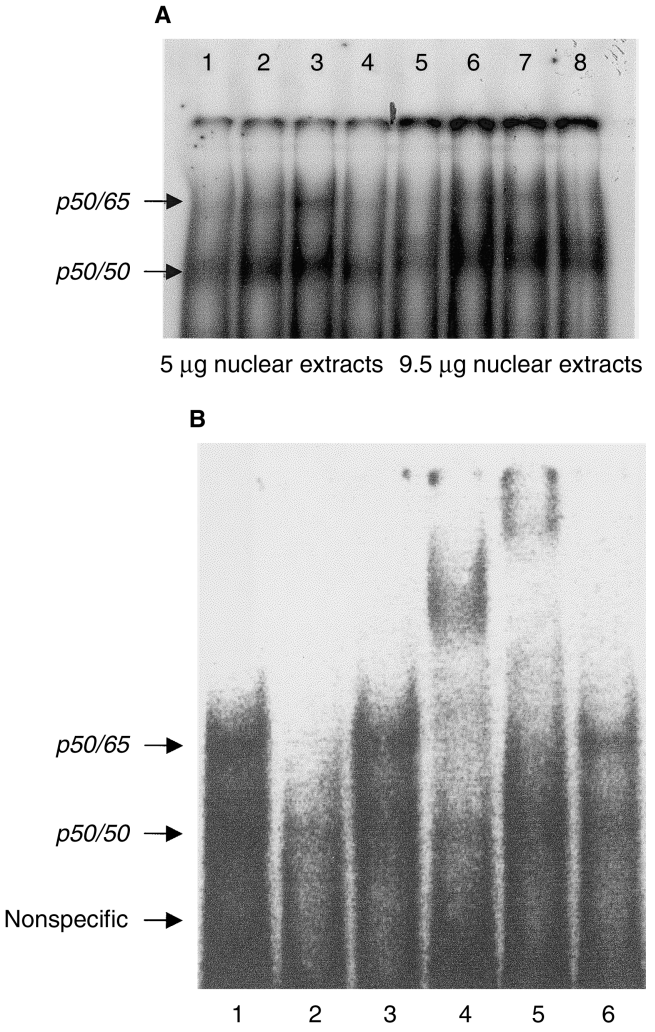

Fig. 10. EMSA and supershift assay for NF-κB. (*A*) EMSA for detection of NF-κB subunits was performed using 5 μg (lanes 1-4) and 9.5 μg (lanes 5-8) nuclear extracts. Results show increased NF-κB (p65 and p50 subunits) binding in nuclear extracts of the LC-1 (lanes 2, 6) and LC-2 (lanes 3, 7) treated cells compared with nuclear extracts of the control (lanes 1, 5) and HSA (lanes 4, 8) treated cells. (*B*) Supershift assay. Nuclear extracts from cells treated with LC-2 were incubated with NF-κB specific DNA probe alone (lane 1) or in presence of 30-fold molar excess of a specific competitor (wild-type, 42-bp) oligonucleotide (lane 2) or a nonspecific competitor (mutant) oligonucleotide (lane 3). Antibodies for the supershift assays: p50 (lane 4), p65 (lane 5), and c-Rel (lane 6). This experiment is representative of two and shows participation of p65 and p50 subunits.

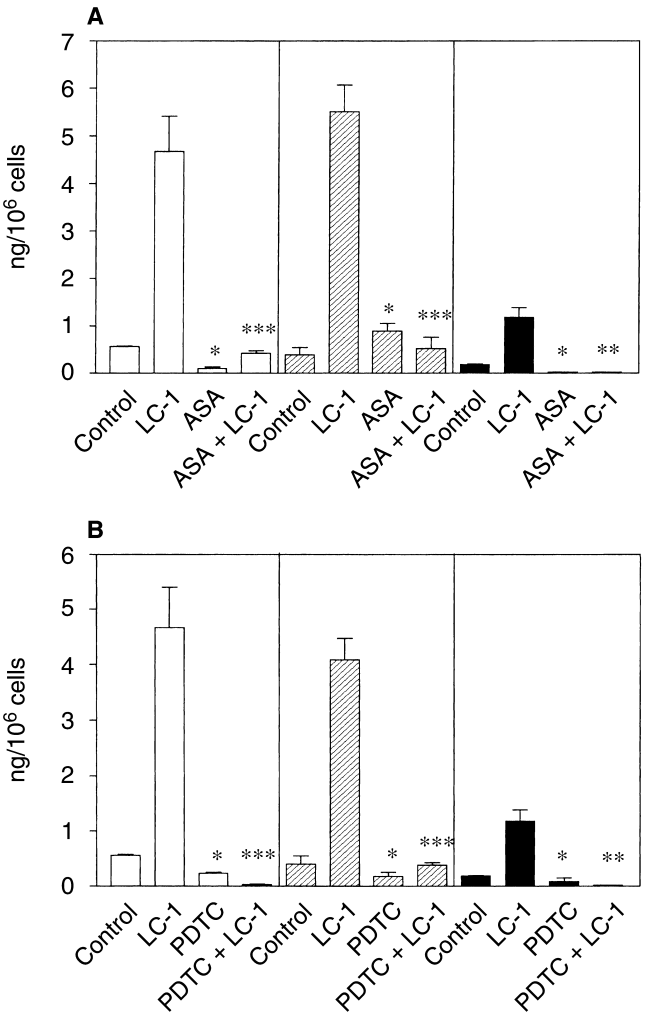

Fig. 11. Effects of aspirin (ASA) and pyrrolidineditiocarbamate (PDTC) on LC-1 stimulated IL6, IL-8 and MCP-1 production. Production of IL-6 (□), IL-8 (▨) and MCP-1 (■) were inhibited by aspirin (20 mmol/L; *A*), and PDTC (12.5 μmol/L; *B*), both inhibitors of NF-κB. Neither ASA alone nor PDTC alone had significant effects on cytokine production. Results are presented as mean ± SEM (***$P < 0.001$ vs. LC-1, **$P < 0.05$ vs. LC-1, *$P > 0.05$ vs. control).

rate cytokine responses (Fig. 11) confirm the role of NF-κB in LC-induced cytokine response. This observation lends further credence to the protein-overloading hypothesis and identifies a step in the pathogenesis of myeloma nephropathy potentially amenable to therapeutic interventions.

Strikingly similar effects of LCs isolated from patients with amyloidosis and LC deposition disease (LCDD) on mesangial cells were described by Russel et al [42]. These investigators observed that although tubulopathic LCs had no effect, glomerulopathic LCs were either internalized or bound to the surface of mesangial cells and caused dramatic cytomorphological alterations or elicited inflammatory response including production of MCP-1 and PDGF-β through activation of NF-κB. These studies shed significant insight into mechanisms of LCDD and LC amyloidosis, and show that different LCs utilize similar pathogenic pathways in different cell populations. Our LCs were isolated from patients with minimal or no albuminuria and no clinical evidence for any glomerular disease. These observations show that different LCs may utilize similar inflammatory pathways on different cell types resulting in different consequences.

The cytokine responses observed here suggest a prominent role for IL-6. IL-6 was originally identified as a

26 kD protein in supernatants from antigen- or mitogen-stimulated mononuclear cells, which induced immunoglobulin synthesis by activated B cells [43–45]. Later, IL-6 production in many other cell types including glomerular mesangial and PTCs was reported in response to lipopolysaccharide, IL-1 and TNF-α [46–48]. IL-6 has pleiotropic proinflammatory effects in many cell types. It is the principal mediator of acute phase response and has an accessory role in T cell activation. The possible roles of IL-6 in disease have been shown dramatically in transgenic mice constitutively expressing high levels of IL-6 [49, 50]. These mice develop fatal plasmacytomas with membranoproliferative glomerulonephritis and massive bone marrow infiltration, displaying many similarities to multiple myeloma. Malignant plasma cells in multiple myeloma both produce and respond to IL-6. Although it has a very short half-life, high amounts of IL-6 are detected in the plasma and urine of patients with multiple myeloma and renal transplant rejection [51, 52]. The IL-6 gene in chromosome 7p21 has been shown to have multiple functional response elements in the 5′ promoter region. Consensus sequences for the glucocorticoid response element, activator protein-1 (AP-1) binding site, cyclic AMP response element as well as NF-κB binding site have been identified [45, 46] on this gene. It is of interest that the IL-6 promoter is strikingly similar to the regulatory region of the c-*fos* gene, another transcription factor intimately linked to renal ischemia. The increased proximal tubular production of IL-6, in addition to the elevated levels contributed by the malignant plasma cells in myeloma, are suspect participants in the pathogenesis of chronic tubulointerstitial disease seen in multiple myeloma. The precise role of IL-6 in this process and whether it is predominantly regulated by NF-κB or by other transcription factors, such as NF–IL-6, activator protein-1 (AP-1) complex, remain to be resolved.

Our observations have implications beyond renal involvement in myeloma and may pertain to the enigma of progressive nature of chronic renal insufficiency in all proteinuric states. All proteinuric kidney diseases associated with significant albuminuria necessarily have impairment of the glomerular charge-size barrier to plasma proteins. Compared with albumin, myeloma LCs are more freely filtered and in healthy individuals significant quantities are filtered and endocytosed by PTCs. In disease states with altered glomerular permselectivity, the glomerular basement membrane would be even more permissive to LCs than in the healthy state. Thus, PTCs would be more likely to face larger quantities of LCs, which have higher affinities than albumin for the endocytic apparatus of the PTCs [19, 34]. The observation that diabetic patients excrete significant quantities of κ-LCs is in agreement with this thought [53]. It is plausible that low-molecular-weight proteins that accompany albumin predominantly mediate the protein overloading in albuminuric states. Immunoglobulin LCs are the most abundant proteins among this group of proteins that are likely to mediate such cytokine responses leading to chronic tubulointerstitial inflammation either as a primary event or as a process responsible for progression of kidney disease in proteinuric states.

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